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THE PHYLLOSPHERE

I. AN ECOLOGICALLY NEGLECTED MILIEU

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INTRODUCTION

In an ecological analysis of the habitat of epiphytes, carried out in Java, Indonesia, attention was drawn to the rhizosphere of the epiphyte [42]. Observations were made on the root systems of the fern *Drymoglossum pilloselloides* Pres land some orchids, all commonly occurring on the trunks and branches of trees, but often growing on leaves. In addition to the symbiotic root-inhabiting fungi a number of other, mostly saprophytic, soil fungi and fungi regarded as weak pathogens could be isolated. A few characteristic colonies of yeasts and bacteria appeared regularly. *Cryptococcus* and *Beijerinckia* species, and *Mycoplana rubra* De Vries and Derx were obtained in cultures.

The occurrence of soil bacteria in such situations is not surprising. The root system of epiphytes is a natural trap for air- and water-borne micro-organisms and the reaction (pH 5.5) of the medium used for the isolation of the fungi is favourable for the growth of the epiphyte rhizosphere bacteria. Beijerinckia is abundant in acid tropical soils. Thus, it is not astonishing that the humus-rich substrate, formed by the decomposition of leaf litter and senescent and sloughed-off periderm cells of the trees on which the epiphytes grow, provides a suitable environment for Beijerinckia. Moreover, the foliar exudates which are washed down the crevices of the bark provide a supply of nutrients sufficient to maintain a complex community.

Extending this consideration to the actual source, the foliage, of this downward nutrient stream, it is similarly obvious that the

leaf itself, provided that it is mechanically strong enough and has a sufficient length of life, should be able to support the growth of epiphytes. Dust and plant debris collect between the roots of such leaf epiphytes and these in turn would provide an environment for micro-organisms.

As a preliminary attempt to investigate whether the occurrence of these micro-organisms is dependent on the rhizosphere of the epiphyte, comparative platings were made from the epiphyte root system and an adjacent area of the supporting leaf, which was not covered by the epiphyte.

In a preliminary note [43] the author has reported the preponderance of fungi over bacteria in the rhizosphere, a condition which persisted throughout the various stages of development of the supporting leaf. On the other hand the initial predominance of bacteria on leaf surface not occupied by the epiphyte alters with age, so that on senescent leaves fungi apparently predominate. The microbial population, as determined by the use of Burgeff's acid medium, appeared to have a characteristic composition and to contain a limited number of species, with *Beijerinckia* sp. as the characteristic bacterial representative.

The next step was to ascertain whether these organisms could also be isolated from healthy leaves not bearing epiphytes. This proved to be the case. Beijerinckia was isolated in 192 out of 196 cases examined.

The external surface of the leaf, as an environment for micro-organisms, has been termed the *"phyllosphere"* by analogy with the rhizosphere of roots (Ruinen [43]). Based on similar considerations Last [29] suggested independently the same term as a result of a study of the incidence of fungi on cereal leaves. Since then, the term has been accepted (*e.g.* Di Menna [38], Greenland [17], Kerling [27]).

So far, with a single exception, the occurrence of Beijerinckia has been reported to be pantropical. Kluyver and Becking [28] pointed to the prevalence of this bacterium in lateritic soils as a possible explanation of its limited distribution, which, however, is not completely satisfactory. Derx [12] suggested a possible association with the roots of specific tropical vegetation, presumably leguminous plants or rice.

The observation, however, that the leaf surface of a great number

of plants growing in humid tropical conditions offers a suitable habitat for micro-organisms in general and for Beijerinckia in particular still offers another explanation of the widespread occurrence of this bacterium. The probable importance of the nitrogen-fixing properties of Beijerinckia for this environment, justified a further investigation of the phyllosphere. The primary aim of this investigation was to ascertain whether Beijerinckia and, possibly, other nitrogen fixing bacteria occur in the phyllosphere and are characteristic for this habitat. Moreover, it seemed desirable to find out which environmental factor or factors condition this habitat.
The survey made in Indonesia in 1955 was extended during an eight-months stay in Surinam at the Government Agricultural Station, Paramaribo in 1956–1957. In this paper the preliminary findings are presented with a discussion of the problems arising.

MATERIALS

The material consisted of fresh leaves collected at random in different localities of Java, Sumatra, Banka, and Bali. They were picked from trees up to 24 m in height, climbers, shrubs, perennials, and epiphytes growing in the Bogor Botanic Garden, in primeval and secondary forest, and from coastal vegetation and plants growing in craters. At first similar random sampling was carried out in Surinam, material being obtained from wayside and jungle vegetation of the forest floor as well as from the crown of felled trees. Only a part of this botanical material could be identified taxonomically. It contained ferns, along with monocotyledonous and dicotyledonous leaves. Later the leaves of citrus, cacao, and shade trees growing in the experimental plots of the Agricultural Station were selected for a closer study.

In the first instance healthy-looking mature leaves were sampled. Later young and senescent leaves were also included in the observations.

METHODS

The presence and the extent of the phyllosphere population has been ascertained *i.* by direct microscopy and *ii.* by selective culture in nitrogen-free media.

i. Direct microscopy

Microscopical examination of free-hand surface sections of the fresh leaf at medium (400×) or high (800 to 1000×) magnification is easy and fully satisfactory. Staining with phenolic erythrosine or Lugol's iodine solution increases contrast and reveals lipid drops in the organisms and the environment. A disadvantage of this method is that during preparation of the section

part of the microbial population may be lost due to swelling and disintegration of the microbial layer of the phyllosphere. Much depends on the species composition of the microbial population and the weather conditions immediately prior to the sample leaves being taken; a recent or prolonged wetting increases the chances of loss of microbes.

A more attractive method, which is also suitable for the preparation of permanent mounts necessitates infiltration of the microbial layer of the phyllosphere with a solution of collodion or, even better, a solution of water-soluble plastic material. When dry the film is stripped off and mounted for examination. Sometimes part of the microbial layer remains attached to the leaf, particularly so if the layer is thick or anchored by fungal hyphae which have grown into the epidermis. Comparison of a number of preparations yields a satisfactory over-all picture of the composition and development of the microbial layer of the phyllosphere.

ii. Selective culture in nitrogen-free media

a. Sterile, powdered starch scattered lightly over the surface of leaves lying in petri dishes was slightly moistened and the leaves then incubated at room temperature in an atmosphere saturated with water vapour. If microscopical examination after 24- or 48-hours incubation revealed the presence of bacterial cells between the starch grains, samples were plated out on glucose–phosphate or starch agar having a reaction of pH 5.5 for Beijerinckia and pH 7.0 tp 7.2 for Azotobacter. By repeated replating the organisms could be isolated in pure culture.

b. The leaves were immersed in a shallow layer of liquid medium, containing 1% glucose + 0.1% KH_2PO_4 or 0.05% K_2HPO_4 in 250-ml flasks. The former medium yields mainly Beijerinckia, the latter the common Azotobacters. After a few days incubation the various organisms were isolated by the use of agar plates.

At a later stage of the survey in only a few cases selective culture was carried out by the method devised by Derx [10 11], and also used by Jensen[26], which makes possible the simultaneous detection of the various *Azotobacter* species occurring in a sample. The liquid media used had the following composition:

0.1% KH_2PO_4 + 1% glucose	*Beijerinckia* species
0.05% K_2HPO_4 + 1% ethanol + 0.1% $CaCO_3$	*Beijerinckia* spec., *Azotobacter insigne*, *A. agile*, *A. vinelandii*, *A. chroococcum* and others
,, + 1% mannitol + 0.1% $CaCO_3$	*A. vinelandii*, *A. chroococcum*
,, + 1% sodiumbenzoate + 1% ethanol + 0.1% $CaCO_3$	*A. agile*
,, + 1% sodiumbenzoate + 0.5% mannitol + 0.1% $CaCO_3$	*A. vinelandii*

Incubation at room temperature.

RESULTS

General considerations

The findings in Surinam fully confirm those made previously in Indonesia. Well-developed phyllosphere populations are of general occurrence. In addition to Beijerinckia a number of oligonitrophilic and nitrogen-fixing micro-organisms could be isolated by the use of media having a reaction near neutrality. Among these are a number of yeasts and fungi, along with species of *Aerobacter*, *Arthrobacter* and *Pseudomonas*. The presence of these phyllosphere organisms is visible with the naked eye as a patchyness or as a hard gloss on the surface of the epidermis proper, depending on their distribution in scattered groups or in a continuous film. In addition to the mentioned organisms a highly complex population, which often gives a specific colour to the phyllosphere, is usual. Thus, a black or blackish phyllosphere colour will point to the presence of Cyanophyceae or sooty moulds, whereas patches with light bright-green or reddish tinges point to Chlorophyceae, and greyish colours to moulds. The bluish green, white-bordered patches of crust lichens are most conspicuous. Beneath the microbial layer the leaves are dark green unless they are, incidentally, infected by parasites.

The microbial layers of the phyllosphere show marked differences in species composition and surface spread, which are seemingly characteristic for particular plant species. No further attention has, sofar, been paid to this aspect, which offers subject for further investigation.

Not only do different plant species show striking differences in their phyllosphere populations, but different leaves of one and the same plant show variations depending on the position of the leaf in the vegetation and on the age of the leaves. As a rule the increase in population starts after the leaf has reached maturity and may reach considerable dimensions on the older leaves (Plates 1, 2, 7, 8, and 9).

In view of the changes in the population as the phyllosphere layer develops, it is easily understandable that the enrichment cultures give different results for different sample leaves. Particularly in the case of older leaves, samples removed within 24 hours of the commencement of incubation has a flush of bacteria which could obviously grow more rapidly on the combined nutrients and the

added substrate than the Azotobacteriaceae, and were therefore, more easily purified. After prolonged incubation of the leaves (2 to 5 days) in the presence of the nutrient medium *Azotobacter* spp. and *Beijerinckia* spp. appeared. On the other hand, in the case of young mature leaves, the latter species could be found to have multiplied abundantly after eight hours of incubation.

The micro-organism population of the phyllosphere

It has been mentioned already that during the survey of the phyllospheres of various plant species in Indonesia, Beijerinckia was found in 192 of 196 leaf samples. The four cases in which Beijerinckia was not found seem comparitively insignificant. They may be due either to faulty manipulation or to the absence of this organism from the phyllosphere population of these particular leaf samples. That an incubation time of 24 hours is either too long or too short for the various kinds of micro-organisms became obvious during the course of the survey. On the other hand the acid reaction of the medium used for the isolation of Beijerinckia may have precluded the development of, for instance, Azotobacter, which, as became apparent later, often form the main component of the phyllosphere population.

In Surinam, a preliminary indication of the presence of potential nitrogen-fixing bacteria in the phyllosphere was obtained by incubation of the leaves in media having either an acid or neutral reaction and to which 1% glucose has been added. Selective cultures in accordance with the method used by Derx were subsequently set up in the case of a few leaf samples. Microscopical examination of the leaf surface was carried out in all cases. These observations covered a wider range of micro-organisms, and a smaller number of higher plant species.

Both direct microscopical examinations and culture methods provided information about the other inhabitants of the phyllosphere community. The data presented below are incomplete as only those organisms which could be readily identified were recorded. Moreover, at high magnifications (800 to 1000×) only fractions of the total leaf surface could be examined and, although an over-all picture could be formed, many details will certainly have escaped observation. Furthermore, it should be remembered that the main object of the investigation was to ascertain the occurrence of nitro-

gen-fixing bacteria in the phyllosphere. These, therefore, received most attention. In addition the culture media used were not suitable for the growth of organisms other than those having minimum requirements for combined nitrogen. Consequently, it does not necessarily follow that the organisms appearing in the table were the only ones initially present in the sample.

The observations made in Surinam are summarized in Table 1. For tabulation the organisms have been separated into groups, *i.e.* bacteria, actinomycetes, fungi, and algae; the bacteria being separated into genera and species in the case of some of the Azotobacters,

TABLE 1

Organisms found by selective culture and direct microscopical observation in the phyllosphere of plants in Surinam

Origin of samples	Number of samples	Bacteria										Actinomycetes	Fungi		Algae		Other organisms		
		Clostridium spp.	*Beijerinckia* spp.	*Azotobacter* spp.	*A. chroococcum*	*A. vinelandii*	*A. agile*	*A. insigne*	*A. beijerinckii*	*Aerobacter* spp.	*Spirillum* spp.		Yeasts	Fungi	Chlorophyceae	Cyanophyceae	Lichens	Mosses	Prozotoa
Random samples of leaves from virgin forest	15	4	15	14	4	7		2		9	11	15	9	15	8	2	3		11
Random samples of leaves from trees in experimental gardens	12		10	11		1			1	2	2		7	12	3	9	7		2
Random samples of Tillandsia leaves	2		2	1					1		2		1	2		1			
Random samples of citrus leaves	24	1	8	24	8	3			2	6	8	2	8	20	14	7	9	1	2
Random samples of Cacao leaves	12	1	11	12	3	2	1				8	1	8	12	12	6	2	1	6

isolated according to the method of Derx, the remainder being enumerated simply as yeasts, fungi, Chlorophyceae, Cyanophyceae, lichens, mosses, and protozoa. A great part of the organisms is highly motile. A detailed report of the findings will be published elsewhere.

Development and extent of the phyllosphere micro-organisms population

It is evident that during the development of a leaf micro-organisms will be absent from the leaf surface for only a short time, if at all. In an established plant community the presence of an abundant phyllosphere population of micro-organisms precludes the absence of micro-organisms on the surface of the developing leaves. Actually, bacteria and hyphal fragments have been observed even on leaves at the earliest stage of development; these are obviously the result of infection via rain spattering or dew dripping from leaves at a higher level (Plate 3). The nutrients in this water are sufficient for only a limited number of divisions of these microbes. Vigorous development of the organisms occurs during the maturity of the leaf when it is photosynthesizing and transpiring actively.

At this stage competition between the various types of organisms in the inoculum commences. At first the nitrogen-fixing organisms are in a favourable position since they are independent of combined nitrogen and not exacting as to the source of energy. They have been observed to multiply in the capillary water held by surface structures, *e.g.* trichomes, depressions in the epidermis overlying the anticlinal cell walls, and later in the vicinity of fungal hyphae where nitrogen is, or becomes, insufficient for the growth of the latter (Plate 3 to 7).

Plate 3 shows one of many minute colonies found on a young Qualea leaf from the canopy of virgin forest after a recent infection. In the drop of fluid a small colony of cocci has developed, whereas there has been no comparable development of bacilli. In the case of an older leaf (Plate 4) secondary colonies and hyphae have developed on top of the continuous layer of bacteria covering the surface. In Plates 5 and 6 of *Coffea* sp. and *Pellionia* sp., respectively, the epidermal cells are clearly outlined by localized growth of micro-organisms in the depressions. This can be considered an indication of the stage at which exudation from the leaf commences. After this stage the micro-organisms multiply actively and cover the entire leaf surface, and the layer subsequently grows in thickness.

Eventually the nitrogen-fixing bacteria will supply the necessary combined nitrogen for the development and maintenance of the other members of the phyllosphere population. The physiological state of the supporting foliage will ultimately be the factor deter mining which of the micro-organisms will become dominant in the phyllosphere.

Plate 1. Cacao leaves of various ages. On the left a recently mature "clean" leaf; on the right a senescent leaf with conspicuous patchy growth of lichens and with algal growth along the veins. The leaves between show intermediate stages.

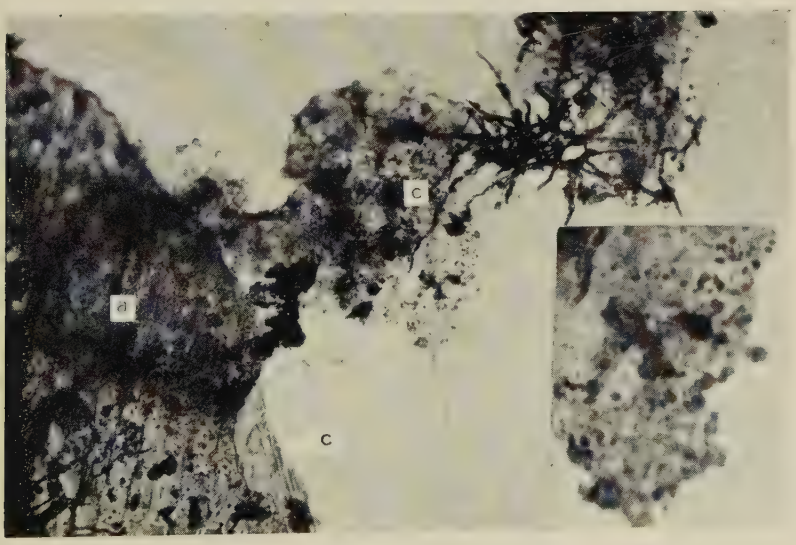

Plate 2. Surface section of a leaf of *Eria* sp. from Sumatra. At *a* the epidermis is covered with the phyllosphere population, at *b* the leaf epidermis is bare, at *c* the microbial layer is hanging free in the preparation. In the microbial layer yeasts, fungi, and algae are visible in the binding mass of Beijerinckia cells (see insert) (300×; insert 700×).

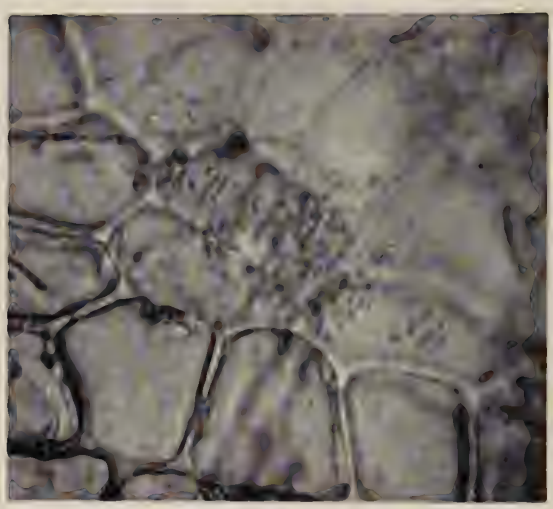

Plate 3. Appearance of the surface of a young leaf of *Qualea* sp. from the canopy of the Surinam virgin forest. Initial development of diplococci in a primary mixed colonization in a well-contoured drop of dew (700×).

Plate 4. The same as Plate 3, but at a later stage. On the surface of a thin but continuous layer of bacteria secondary micro-organisms have developed in flush in drops of water and in capillary water along the hyphae (525×)

Plate 5. Appearance of the surface of a young mature leaf of *Coffea* sp. from Java. Microbial growth, mostly *Beijerinckia* spp. along the depressions over the anticlinal walls of the epidermal cells (700×).

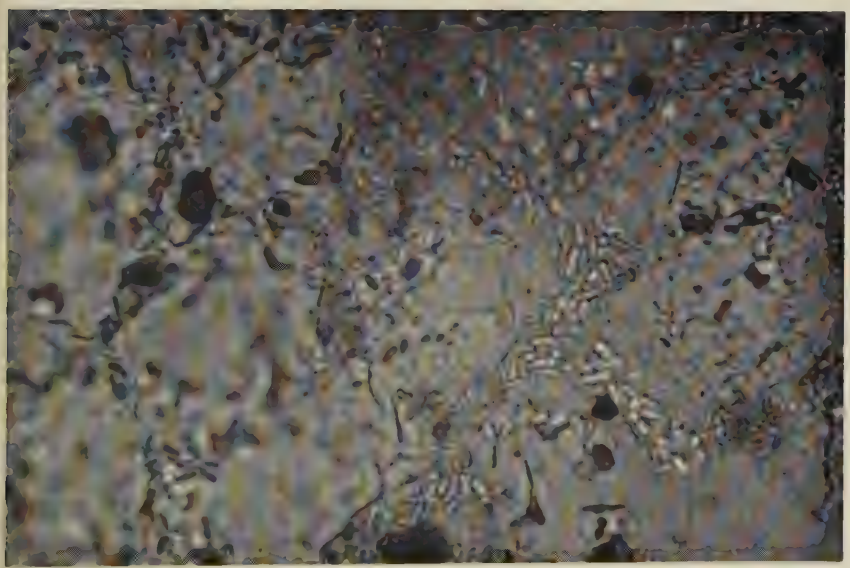

Plate 6. Surface of leaf of *Pelliona* sp., a common plant of the forest floor in West Java, from a plant grown in the greenhouse of the Leiden Botanic Garden. Growth of micro-organisms; Azotobacter, *Beijerinckia* sp., actinomycetes, and yeasts spreading from the depressions over the anticlinal walls of the epidermal cells over the intervening projections of the cells (700×).

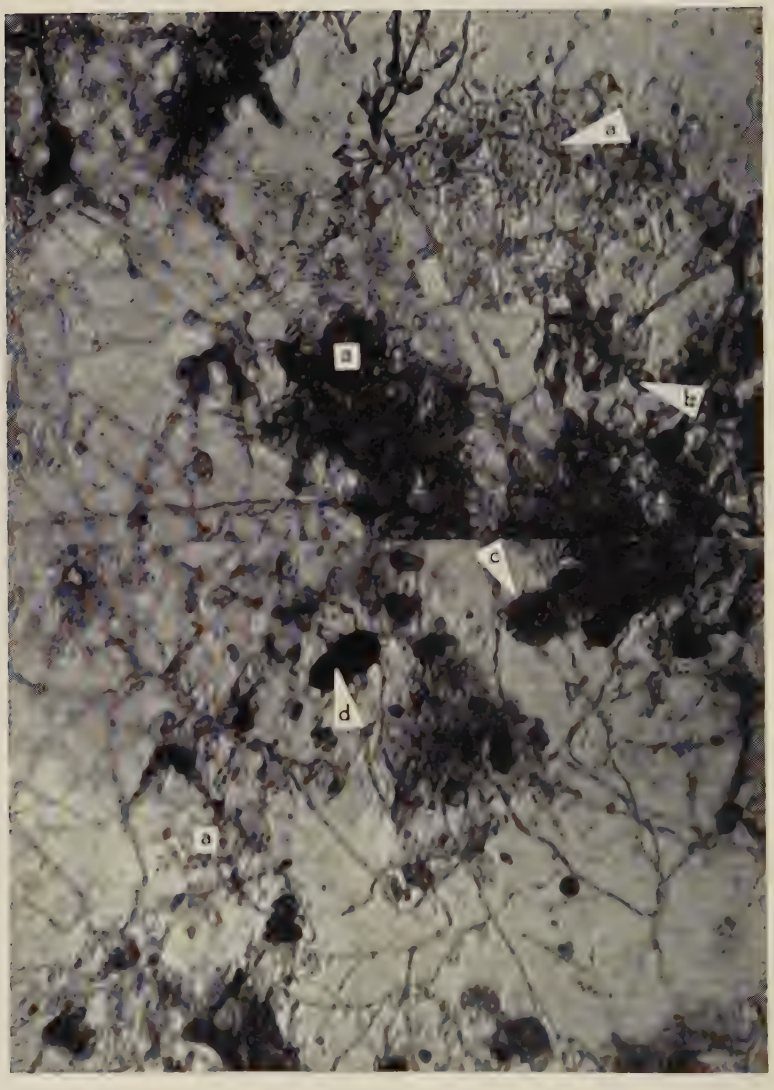

Plate 7. Collodion film with embedded elements of the phyllosphere population from a mature leaf of *Citrus* sp. from Surinam. Only part of the population has been removed in the film. In the upper left hand corner the impression left by bacteria and hyphae on the surface of the film can be seen. In the centre a patch of algal growth can be seen on top of the layer of Azotobacter (*a*), *Trentepohlia* sp. (*b*), and a young thallus of Phycopeltis (*c*). There is a net of fungal hyphae and a scattering of pollen grains over the whole surface, a fungus sporangium can be seen at *d* (525×).

In the specimens examined the microbial layer covering the leaf surface varied from 1 to 22 μ in thickness, the projecting filaments of algae, fungi, and lichens not included.

It is frequently considered that the magnitude of the bacterial population is an important indication of the fertility of the soil. For comparison of phyllosphere populations with soil populations counts of bacteria on the leaf surface of a mature cacao leaf were made.

The leaf sample was collected in the Government Experimental Gardens in Paramaribo after a period of continuous rain (31 December 1956 6.5 mm; 1 January 1957 61.2 mm). Loss of bacteria during preparation of the material could not be prevented due to the water-logged state of the microbial layers.

In addition to a well-developed hyphal net larger organisms were present, *e.g.* Chlorella and Scytonema. The bacteria filled the spaces in the network and were concentrated in thick clusters round the algae (Plate 8). The counts were made at some distance of the algae, where the bacterial layer was only two or three cells thick. The estimate obtained may therefore be expected to be on the low side.

The density per square of $8 \times 8\,\mu$ varies from 6 for the larger Azotobacters to 17 for Beijerinckia. Taking a mean of 8 bacteria per square the population amounts to 12.5 millions per square centimeter *.

The ready appearance of a number of oligonitrophilic organisms within 24 hours after inoculation of a nitrogen-deficient medium with leaf material suggests that these organisms are either actively growing or in a quiescent condition on the leaf surface, such that they respond readily to favourable changes in the environment. In this connection the following observation seemed remarkable Moisture collected in the morning from cacao leaves in Surinam was highly viscous, turbid and of a yellowish green colour. Microscopical examination showed it to be teeming with highly motile organisms (bacteria and some flagellates), and with less conspicuous microbes (yeasts and algae). Inoculation of this fluid at a dilution 1 : 1000 into an ethanol-phosphate medium gave rise to a

* Compared with Azotobacter counts for fertile soil of the temperate regions (from 50 to 100 thousand/gram soil) and with the computed total microbial numbers per gram of soil in tropical forest, *e.g.* 500,000 in Malaya (Corbet [8]), 2219 to 2820 millions in Kenya (Meiklejohn [37]) or 2,860,000 in Brazil in a citrus plantation (Döbereiner [13]) the figure is impressive.

yellowish gray turbidity within four hours, due to bacteria, some yeasts, and flagellates. Direct examination of the leaf showed the striking development of a multi-layered population of micro-organisms on the leaf surface. It has to be mentioned that the sample had been collected after two days without appreciable rainfall, *i.e.* 1.7 and 0 mm, respectively, and with heavy dew during the nights.

Observations on other leaves of the cacao and of other plant species yielded similar results.

As has been mentioned before, the kinds of micro-organisms occurring in the phyllosphere populations and the degree to which the leaf surface is covered vary for different plant species. Cacao leaves from the experimental plot of the Agricultural Station at Paramaribo appeared to be more readily colonized by Beijerinckia than by the species of Azotobacter, whereas the latter apparently predominate on citrus leaves. Re-examination of cacao and citrus leaves from plants growing in different localities is needed since such differences in the predominant species may well be as much the result of an initially heavier specific infection, as the result of a selective habitat offered by a specific plant, either as the consequence of its nutritional status or its specific exudates. Moreover, the location within a particular plant community proved to be of importance.

Along with the increasing excretion of substrate material from the leaf at the time when the leaf reaches maturity nutritional conditions for colonization improve and other micro-organisms follow the pioneers. The release of the metabolites and autolysis products of these organisms further influences the nutritional environment. Plate 1 shows the gradual colonization of the surface of a cacao leaf in which the larger elements such as algae are becoming increasingly conspicuous.

Summarizing the findings from direct observations and from cultures it appears that the following sequence occurs (see Fig. 1).

The phyllosphere is first colonized by bacteria, actinomycetes, and fungi. When colonization commences, the oligonitrophilic bacteria have an advantage due to their less exacting nutritional requirements. Moreover, many of them are motile and they spread rapidly over the leaf surface when it is wet. The nutrients supplied

by the leaf exudates and by the Azotobacter population provide an adequate substrate for successive colonizers; algae, yeasts, and lichens appear and a mixed population of other unicellular organisms such as flagellates, amoebae, myxomycetes, and ciliates develops, which feeds on the other micro-organisms. Arthropods may follow in due course.

This is generally the end of the succession. Under conditions of adequate humidity, temperature, nutrition and longevity of the leaf, however, mosses, ferns, and occasionally phanerograms, well-known epiphytes, may develop. Such a much expanded phyllosphere population presents conditions for colonization by still more micro-organisms in the tangle of roots and on the leaflets of the epiphyllic vegetation. A rhizosphere population will now develop as a result of the development of this new vegetation and the bacteria will colonize the additional leaf area and occupy the phyllosphere of these later immigrants.

The climax population of the phyllosphere as described above will be reached and maintained on the mature and fully active leaf. Later, and especially as metabolism of the leaf and exudation into the phyllosphere decrease during senescense, the microvegetation will gradually become more autonomous. The nutritional requirements will be met by mutual exchange between the various organisms of the phyllosphere and from leachings from leaves situated at higher levels in the plant community. At this stage the leaf functions chiefly as a mechanical support.

It may be mentioned that the juvenile stage of the leaves lasts for only a couple of days up to a week for many species, whilst the

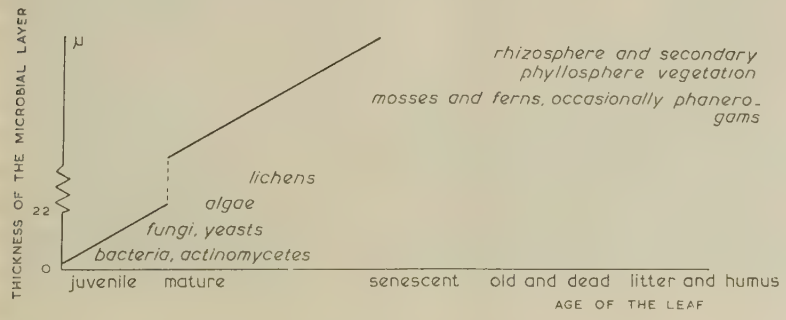

Fig. 1. Development of and sequence in the phyllosphere population during growth and senescence of the leaf.

period of maturity may be extended in many non-deciduous species up to a couple of years.

The above scheme representing the conditions prevailing during the development of the phyllosphere population is suggested.

The phyllosphere as an environment

After it had been ascertained that microbial growth occurred in great, although variable abundance on the leaf surface the next step was to look which conditions favour the development of the micro-organisms and make the phyllosphere, as it is by definition, an environment particularly suited for microbial life. It proved to be a typically aqueous millieu which is continually fluctuating in volume and in nutrient content.

In the foregoing paragraphs the role of the water has been mentioned already. It offers the life space as well as the medium of exchange with the leaf and the other members of the community. Its quantity, the time of day, and the duration of the wetting all have a considerable influence upon the growth of the micro-organisms, and the composition of the population. Obviously stagnant fluid, *e.g.* in the form of drops of dew, causes an increase in the population which is strictly confined to these drops. As has been mentioned before a damask-like texture is produced in the phyllosphere, due to well-localized, slightly thicker layers of organisms after the evaporation of the water and its absorption by the leaf. On the other hand, rainwater, which spreads evenly over the epidermis produces a microbial layer of uniform thickness; when dry this layer has the glossy appearance of hard lacquer, and is often mistaken for the leaf cuticle. Drainage of the water along the veins causes an accumulation of organisms, also visible with the naked eye (Plates 1, 9).

The variable effect of the water is most clearly observed in the different strata of the green vegetation. The leaves exposed to the sky and prone to the direct wetting by dew and rain show the above sketched features. However, the parts of the leaves which are overlapped by other leaves, as is often the case in large-leaved species, lack the specific gloss or patchyness, even in the outer layers of the vegetation. The differences are still more pronounced beneath the canopy there where during the dry season direct wetting is irregular and occurs only occasionally by the drip of rainwater

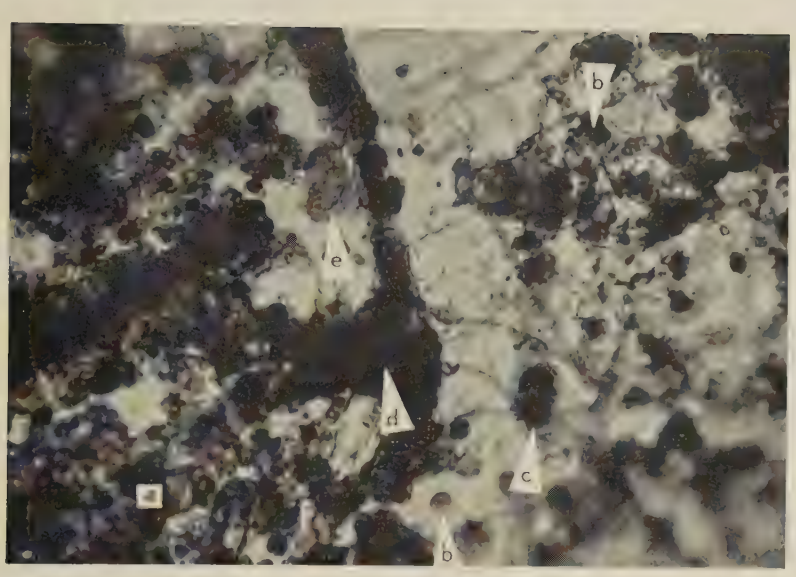

Plate 8. Part of phyllosphere population from surface of mature cacao leaf from Surinam embedded in collodion film. The following organisms can be observed. Groups of bacteria (*a*), yeasts and unicellular algae (*b*), and hormogonium of a lichen (*c*). In the centre of the photograph a filament of Scytonema (*d*) and a protozoon (*e*) can be seen (525×).

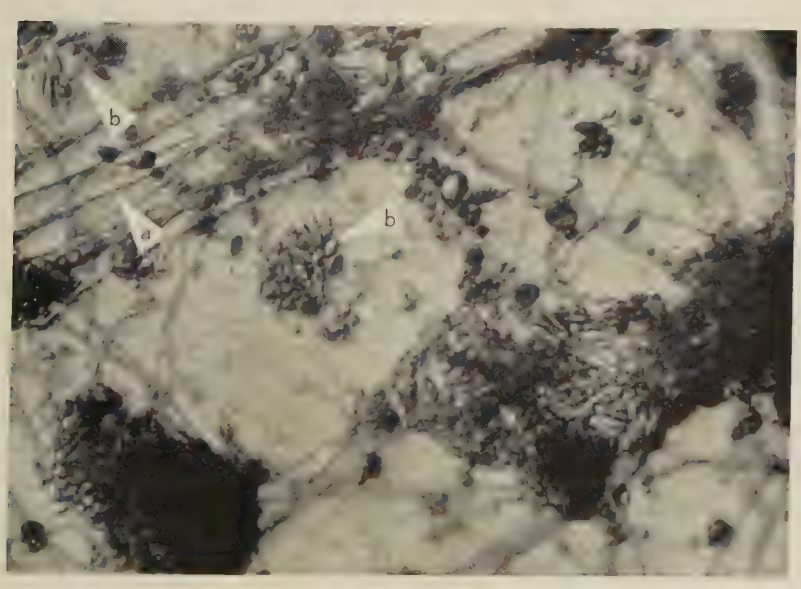

Plate 9. Hyphae growing in the depression above a vein of a cacao leaf (*a*). The masses of ellipsoid cells at (*b*) are unicellular algae (700×).

and dew from the higher levels of the vegetation, or by condensing water vapour from the soil. The resulting microflora, with a preponderance of hyphomycetes and lichens is indicative for the microclimate of high relative humidity but irregular wetting. In the places where a regular run-off from the higher strata occurs, a completely different and more hygrophilic micro- and macrovegetation develops. Many of the organisms concerned have a high rate of division and a light dew suffices for active multiplication to take place (*cf* p. 88). The organisms which divide more slowly lag behind, especially so, if the wetting is of short duration, as is often the case with rain falling during the day. In Indonesia as well as in Surinam locally wetting by dew and ground mist during ten to twelve hours a day is common. In the early morning the vegetation is dripping wet and in the shade the dew remains visible till ten o'clock a.m. Relevant figures about the seasonal incidence and the duration of wetting have not been gathered, as the influence of the water on the accumulation of the phyllosphere organisms only became clear during the course of the observations. They are subject for a future research.

The wettability of the epidermis proved to be another important factor in the development of the phyllosphere population, which may be due to the transfer of nutrients from the leaf to the phyllosphere, and *vice versa*. The fluctuation in this property in relation to the age of the leaf is a common feature. It has been noted by the author that the young leaves of many tropical plants pass from a stage where they are easily wetted and readily absorb water, through a short stage of complete unwettability, which precedes maturity, to a stage of increasing wettability again. In the initial stage close-standing trichomes, as *e.g.* on cacao leaves, hold the water capillary. The wettability post maturity seems to be more complex and may be associated with the weathering by atmospheric conditions and the activity of micro-organisms. The ectoplasmodesmata, which were found to be microscopically visible in many leaves, may be involved in the processes.

The species composition of the phyllosphere population, and the climatic conditions in existence prior to the wetting, influence the uptake of water. Thus, algae and bacteria increase whilst fungi and lichens decrease wettability. The excretion of lipids by the latter

micro-organisms plays a role in determining the surface run-off at the start of rain.

The nutrients in the phyllosphere are furnished by the green plants in the form of foliar exudates. They contain mineral and organic compounds, the latter being characteristic for the different plant species. At a later stage the metabolic and autolysis products of the developing phyllosphere population contribute to the complexity of the composition and the fluctuations in the nutrient medium, whereas sucking insects by their excrements and by stimulation of the excretion of organic material from the leaves cause a further increase.

In the short time available in Surinam only a few analyses have been carried out on the mineral content of dew on cacao leaves. Here the level of potassium appeared to lie at about 80 mg per litre; of total and dissolved nitrogen at 45.5 mg and 0.84 mg per litre, respectively; of total and dissolved phosphorus at 26 mg and 0.36 mg per litre. It is evident that the first figures represent for the larger part the nitrogen and phosphorus locked up in the phyllosphere population. No observations have been done in Surinam on the organic matter present on the surface of the leaf. Analyses made by the author in Holland show that *e.g.* sugars and organic acids are present on the leaf surface in biologically considerable amounts, which fluctuate between wide limits due to climatic conditions and the time of day. The investigation is still in progress.

The soil plays a major role as the source of minerals influencing metabolism of the green plant and the supply of phyllosphere nutrients exuded at the leaf surface.

DISCUSSIONS AND GENERAL CONSIDERATIONS

As the layer in contact with the leaf and with the atmosphere the phyllosphere is subject to the combined influences of both. From the former it derives its nutrients, the leaf exudates and leachings; from the latter it obtains water enriched by dissolved gases and salts. The developing phyllosphere population in turn exerts an influence on the underlying leaf by covering the surface, and by metabolic and autolysis products from the microbial population.

The nutritional status of the plant and, consequently, the mineral

and organic constituents of its leaf exudates are largely determined by the soil. The soil must therefore be included as a factor influencing the properties of the phyllosphere as an environment for the phyllosphere population. The whole community comprising the plant plus the population of micro-organisms of the phyllosphere is influenced by the environmental factors – light, temperature, and humidity – which show in addition to the seasonal variations diurnal fluctuations between physiologically considerable extremes. The daily variation in water supply, whether as rain, or condensed water vapour from soil and atmosphere, is the most important environmental factor for the phyllosphere vegetation. The exudates of the leaf and the soluble products of the phyllosphere population are dissolved and the survivors of the dry spell during the daytime are able to develop in this fresh supply of nutrients. On the other hand the leaf can then absorb this water to offset the losses by evaporation during the daytime. Along with the water, nutrients are taken up from the phyllosphere.

The intermittent exchanges between leaf and the phyllosphere result in an increase in the population of micro-organisms which gradually alters the conditions in the phyllosphere and leads through a slowly changing dynamic equilibrium to a climax in the microbial cover.

The occurrence of a microbial population rich in nitrogen-fixing organisms in the phyllosphere of a vegetation may be of decisive importance for the nutrition of that vegetation. Of the numerous papers in which some of the problems touching this field have been treated only a few will be mentioned in support of the above considerations.

1. *Water relations*

In a given edaphic zone and topographic site diurnal fluctuations in temperature and relative humidity within a vegetation are greatly influenced by the density and species composition of the plant community. The largest variations may be expected at the contact surface between the air and the vegetation. Here again the relief of the canopy, the form and the size of the individual plants modify spatially the pattern of the microclimate. Within a distance of a few meters, measured vertically or horizontally, considerable differences may occur. In a growing vegetation and expanding

canopy the microclimate in the phyllosphere shifts, therefore, from an outer zone with wide variations between complete wetting during a part of the day and complete desiccation during the rest of the time under insolation, to a not exposed region with lesser variations.

In the moist tropics the daily variations of the relative humidity of the atmosphere over the canopy may present a considerable range. Fluctuations from 40 to 94 per cent are common, and deposition of water may occur during most nights. Data on the actual incidence of dew are still scarce and vary considerably with the observation post. Ringoet [40] mentions an annual mean of 249 nights in Yangambi (Congo) with an absence during the rainy season. Zehnder [54] reports 254 nights for the hills covered with primeval forest of South Cameroon and 40 nights of dew fall in the savannah region of the north, whereas Schweizer [44] mentions 208 dew nights for Djember in East-Java. On mountain slopes and in the neighbourhood of extensive water surfaces, such as the sea, lakes or swamps deposition of water from the atmosphere may occur even more often: thus, Holttum [24] records nightly dewfall for Singapore. On the other hand Zehnder mentions an incidence of 150 dew nights on two stations on the coast. The quantities measured equal 0.2 to 0.3 mm of rain (Schweizer [44], Visser [51]). Comparing these recordings by the Leick and Duvdevani methods with those on a drosometer of his construction Masson [35] points out that the actual value may be easily 1.7 times higher.

The active transpiration during the day leads to a loss of water bringing the leaves to wilt, and necessitates an active intake of water to supply the deficit. According to Went [52] absorption of atmospheric water by the leaves begins when the relative humidity exceeds 93 per cent, a level which is regularly reached after the drop in temperature towards or during the evening. The amount of water absorbed by the leaves and transported to the plant body may be greater than the losses by evaporation during the day and part of it is then exuded again after some hours by the root or by the leaves. Thus the intake of water may become a major function of the leaf (Breazeale *et al.*[5 6], Went [52]).

Variations in absorption of water by the leaves of different species due to the structure and the age, have been reported. For instance Ringoet in his study of the water balance of plantation crops mentions an increase of 10.9 per cent in the fresh weight of young

Elaeis leaves exposed to dew compared with 17.9 per cent in the case of old leaves. Such a quantity amounts to *ca* 10 litres of water per night for a tree having approximately 67 kg of leaves. For intact cacao plants the mean intake of dew water amounts to 8 per cent of the fresh weight of the leaves. Ringoet noticed, moreover, that the water absorption continues after sunrise. This is a point of interest, as it may be expected that due to the rising temperature the physiological activity of the phyllosphere population will increase, and that a steady uptake by the leaf of organic matter released in the medium by the microbes will continue until the water has completely disappeared. It forms subject for future research.

The variations in the wettability of the leaf surface in a diurnal rhythm, and in relation to the age of the leaf and the environmental factors have been the subject of a number of papers (Fogg [15 16], Linskens [31 32 33]). The decreasing wettability during the prematurity stage is apparently connected with the exudation of lipids and waxes at the leaf surface. The subsequent increase after the leaf has ceased to grow seems to be due to the continued excretion of organic acids and minerals and the resulting hydrolysis or saponification of the first formed wall constituents. Moreover the action of cutin-consuming micro-organisms may enhance the breakdown of the cuticle. The unabsorbed dew is the environment in which the phyllosphere population develops on the material excreted by and leached from the leaf. It appears of importance here, that the evaporation deficit of the day may cause an increased permeability of the leaf tissue, with the result that considerable amounts of minerals and organic matter are deposited on the leaf surface. According to Maximow [36] these are lost to the plant even when the wilting is reversible. It seems, however, more probable that in the humid tropics at least a part of such excretions may be re-absorbed.

2. *Nutrients in the phyllosphere*

The occurrence of a rich microbial population in the phyllosphere is in itself proof of an environment with a considerable, if not specific supply of nutrients; the abundance of epiphytes in the moist tropics or elsewhere under perhumid conditions – even if these only occur for part of the day – is another indication. In this con-

nection attention may be drawn to the Bromeliad *Tillandsia usneoides*, which covers the trees in the American tropics and subtropics, obviously living on plant excretions but which can also be selfsupporting on aerial wires. An exceedingly rich micropopulation has been observed on the leaves of this plant by the present author.

The data on the excretions of plant nutrients by leaves have vastly increased since Arens [1] and Lausberg [30] re-introduced the topic in 1934. The losses from field crops and trees due to leaching by rain are well known and have lately been studied in more detail both qualitatively and quantitatively. Recently renewed investigation into the inter-action between higher plants by way of excretion products has made clear the importance of a number of these substances (Grümmer [18]). In part they seem to be highly specific for particular plant species. The age of the leaf and its physiological condition at the time of or prior to the wetting determine the composition and the quantity of the exudate. It has been well established that nutrients are more readily leached from mature leaves and such approaching senescence (Arens [1], Tukey *et al.*[34 50]).

The exudates contain both mineral and organic materials, the latter sometimes being twenty times greater in quantity than the former. The losses by leaching may be severe; they are directly correlated with the leaching time. Tukey *et al.*[50] mention a loss of organic matter of 4.8 per cent of the dry weight of the leaves during 24 hours. With reservations for specific variations and the physical condition of the plant the losses amount from 0.5 to 7 per cent (Engel [14]) to 50 per cent of the ash content of the leaves (Arens [1], Lausberg [30]). Engel's lower figures may be explained by the fact that he rinsed the leaves before making the determinations and thereby removed part of the exuded materials.

The dry weight of the leaves in an established herbaceous vegetation may amount to 30 per cent of its total dry weight, and in a 18-year-old forest stand in the tropics still amounts to approximately 5 per cent (Bartholomew *et al.*[2]). It is clear, therefore, that daily considerable quantities of exudates are deposited on the leaf surface which are available as nutrients for the phyllosphere population. Washed off by rain they are incidentally returned to the soil. Dalbro [9] estimated in the temperate climate of Denmark

the amount of organic matter leached from apple trees to be more than one ton per hectare per annum, of potassium 23.3 to 28.3 kg, and of calcium 6.7 kg. Potassium, sulphates, and phosphates are easily removed by rainwater; this is supposed to play a role in the rapid turnover of nutrients in the vegetation (Hesse [23]).

Noteworthy are Schweizer's data on the mineral contents and sugars in dew and rainwater adhering to the leaf surface and in leachings of leaves of tropical plants. Whereas the ash content in rainwater varied from 66 to 188 mg/l for coffee, from 34 to 764 mg/l for tobacco, in dew the corresponding values varied from 294 to 410 mg/l and 180 mg/l, respectively. For both plant species the values for sugars lie within the range of from 8 to 19 mg/l in rainwater and 115 to 244 mg/l in dew; those for total nitrogen move between 9 and 19 mg/l and 25 and 80 mg/l, respectively. It seems possible that other reducing compounds are included under the heading of sugars, and that the value for nitrogen includes part of the nitrogen bound up in the phyllosphere population. The data are few and no mention is made of the time in the morning, when the dew was collected, a point which may be important in view of the high values obtained for sugar content. It has been ascertained by Tukey *et al.*[50] that the carbohydrate loss from leaves is directly correlated with light intensity and cumulative with the time of wetting. Keeping in mind that a very active microbial life has been observed by the present author in dew collected two hours after sunrise this would mean that towards the end of the wetting period a surplus of energetic material is still present in the milieu, and that the synthesis of microbial bodies may continue till the drying of the surface. Until the same time the uptake of material from the phylllosphere by the leaf will continue.

Schweizer's figures, however incomplete, show the order of variations to be expected, and support the opinion that the dew as such offers a richer medium for the maintenance of a phyllosphere population than rainwater. The few observations made by the present author agree with his data.

The diurnal variations in water content of the leaves has been mentioned above. It is suggested by the present author that nutrients accumulating on the outside of the leaf during the day may be subsequently re-absorbed together with the metabolic and decomposition products from the phyllosphere population during the

nightly wetting of the leaf surface. The results of leaf spraying experiments with radio-isotopes lend still more probability to the above contention (Thorne [48] [49], Tukey *et al.*[50]). The intake proves to be most rapid during the first few hours after spraying, and continues during the entire period when free water is present on the leaf surface. Therefore, the duration of the wetting is doubly important, from the point of view of absorption of nutrients and water by the leaf as well as for the development of the phyllosphere population.

The water and nutrient status of the plant and its phyllosphere population thus appear to be closely interrelated. Dew is obviously more important than rain for the phyllosphere population as all the unabsorbed exudate remains available for its use. The regular rhythm of wetting and drying and the daily supply of nutrients in particular proportions from the leaf induces periodic increases of the micro-organisms which survive periods of drying and which are adapted to this particular kind of environment. As has been mentioned before a succession of organisms follow the gradual change in nutrient conditions resulting from the growing population and the changes in the physiological condition of the leaf.

Dew leads to an enrichment of the leaf environment whereas rain causes an overall loss due to leaching of the nutrients and run-off from the phyllosphere population. Persistent rains may be thus increasingly detrimental. The nutrients in the run-off may remain in the food cycle of the plant being taken up again by the root system, but likely to a lesser extent than by absorption by the leaf. Permanent losses from the site are apt to occur due to leaching and surface erosion of the soil.

What are the implications of the widespread occurrence in the tropics of the phyllosphere organisms, which for a great part are capable of fixing atmospheric nitrogen, on the leaves?

Primarily it suggests, as has been pointed out above, that the vegetation might profit by foliar uptake of the nitrogen bound by the microbial population of the phyllosphere in return for nutrients excreted by the leaves. More research is needed, however, to prove the correctness of the contention that a direct interrelation exists between the higher plant and the epiphyllic microbial flora. It has to be kept in mind that this relationship is different from that

in which symbiotic bacteria are located in special structures on the plant, *e.g.* root or leaf nodules.

Secondly it seems possible that the nitrogen in the soil is augmented by the nutrients and bacteria washed down from the living leaves.

Thus, it might be an additional explanation of the high nitrogen content of tropical soils beneath a plant cover and the recovery of soil fertility during cropped fallow following agricultural crops.

The following considerations may be offered to elucidate the latter point.

The available-nitrogen status of tropical soils is a problem of longstanding importance, as lack of this nutrient is generally the factor preventing repeated annual cropping of the same site. It is now recognized that the exceeding richness of forest soil and of soils under vegetation cover in general, in organic matter and total nitrogen, prevails throughout the tropics, nothwithstanding the fact that the mineral status is often poor, *e.g.* equatorial Africa (Bartholomew *et al.*[2] Greenland [17]), Malaya (Corbet [8]), Indonesia (Hardon [20]), equatorial South America (Jenny [25]), *etc.* The total soil organic matter varies greatly with the type of vegetation, being highest under forest cover – either primeval, secondary or planted – and lowest in the case of bare soil. In between lie the soils under cultivation, with again considerable variation depending upon topography, soil type and structure, treatment received, type and duration of crop and climate. The C/N ratio of the soil organic matter generally varies between 10 and 20. The form in which nitrogen occurs is such, however, that it is not readily available as plant nutrient.

Apart from the combined nitrogen derived from rain, the gains in available soil nitrogen are ascribed to the results of nitrogen fixation by the Rhizobium-nodules of legumes, fixation of nitrogen by free-living bacteria and, finally, to decomposition of soil organic matter. Certainly nitrogen fixation in root-nodules may contribute an important amount especially in those regions where leguminous species are predominant. This is, however, decidedly not the case in all tropical vegetation. Moreover, the formation of nodules, if at all, and the occurrence of effective strains of Rhizobium seems to be exceedingly irregular (Bonnier [4]). Derx [11] suggests that the free-living soil bacterium Beijerinckia may provide the necessary combined nitrogen by association with tropical plant species, presumably legumes, without, however, excluding the non-legumes. The latter trend of thought is also found in Parker's [39] study of non-symbiotic nitrogen fixation in soils not supporting legumes. He emphasizes the importance of the association between higher plants and the potential nitrogen-fixing bacteria, living on root exudates and plant litter, as a major factor in the gains in fertility under grass leys. His argument, based on research in temperate regions, may easily be applied to tropical soils. Previously the importance of the storage of nitrogen in the soil by immobilization in microbial proteins has been stressed

by Harmsen and Van Schreven [22]. In New Zealand Stevenson [46] has pointed out the gains of nitrogen in *Pinus radiata* plantations after 25 years of tree growth, where no other source than the atmosphere appears to be available for the observed accumulation in trees and in the soil. A similar example has been put forward for broad-leaf vegetation and here the accumulation is attributed by Stevenson to symbiotic fixation of nitrogen in the bacterial leaf nodules of members of the Rubiaceae. All these authors envisage the interaction on a large scale of the living organisms concerned.

Birch's [3] research on humus decomposition indicates that the largest proportion of available soil nitrogen is derived from humus, *i.e.* from dead plants. The drying and wetting cycle of the soil is all-important in the release of nitrogen. At the start of the wetting, *e.g.* under field conditions after rainfall, rapid mineralization sets in, which under persistently moist conditions declines within a few days to a lower and constant level, but a fresh release occurs on wetting after every drying. It is important that the degree of desiccation seems to determine the magnitude of the flush of nitrogen, the latter being proportional to the difference in soil moisture content before and after the wetting; moreover, it is also important that in each wetting cycle only a fraction of the nitrogen of the humus reserve is released and, therefore, the reserves persist. A similar rapid release of available nitrogen prior to the increase in bacterial numbers and due to a rapid mineralization after remoistening air-dried soils, has also been reported by Stevenson [47].

It explains the high level of ammonium and nitrate nitrogen in the soil under tree cover compared with that under fallow or rotation crop (Greenland [17], Hagenzieker [19], Hesse [23], and others) and also explains the flush of available nitrogen in the topsoil as the response to the wetting of the soil after rainfall.

All these considerations imply that the magnitude of plant growth, interrelated as it may be with the symbiotic and non-symbiotic fixation of nitrogen, eventually determines the amount of humus in the soil and is therefore a decisive factor in increases of soil fertility.

Dry-matter increments of 12,000 kg per ha per annum are not uncommon for forest stands. Regional variations in this value occur due to differences in vegetation type and environmental factors. The data of Bartholomew, Meyer and Laudelout [2] for forest fallow in Yangambi (Congo) will serve as an example of the order of these increments. Comparisons are made of increment in organic matter, estimated as total dry weight per hectare, and the contents of nitrogen and other plant nutrients in representative samples of leaves, stems *c.q.* wood, roots, dead wood and litter for secondary forest 2, 5, 8, and 18 years old. The trend of these increases for the different vegetative parts is shown in Fig. 2.

It is interesting that whilst the leaf weight remains constant there is a slight but steady increase in root weight and a spectacular increase in the amount of wood, which contains the bulk of the organic matter. The dry matter of the stand contains 701 kg of nitrogen per hectare, 80 per cent of

which had been accumulated within the first 5 years. In addition to the nitrogen locked up in the vegetation there are 1500 to 2500 kg per ha in the topsoil and 3000 to 5000 kg per ha in the layer of soil at 15 to 100 cm depth, for the greater part the product of the forest stand. In comparison, for a

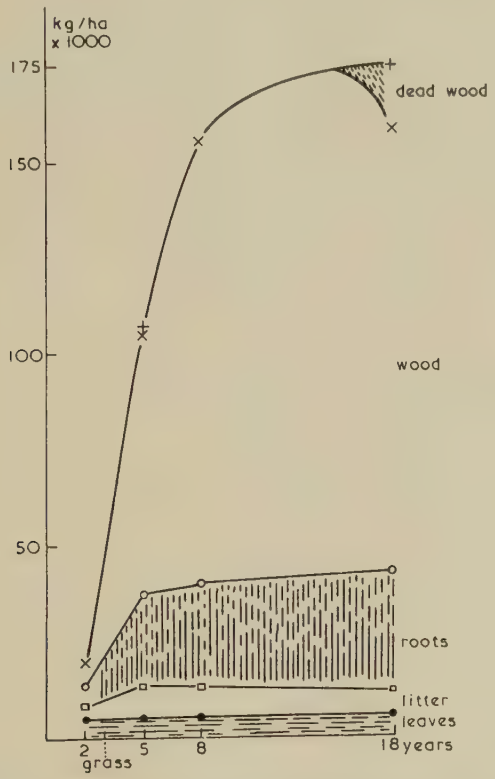

Fig. 2. Increases in dry matter of the vegetative parts of the plant cover in kg of dry weight per hectare, during 18 years of forest fallow in Yangambi (Congo). From the data of Bartholomew, Meyer and Laudelout [2].

Explanation: ● = leaf; □ = litter; ○ = roots;
× = wood; + = dead wood.

cropped soil in the same region the corresponding values are from 1000 to 1500 kg in the topsoil and rapidly decreasing to 350 kg per ha in the layers from 15 to 100 cm depth.

The slow rate of decomposition of the organic matter in the soil under the prevailing conditions of high humidity makes it improbable that the nitrogen liberated in this process would be sufficient for the spectacular growth of the forest, It seems feasible that a more rapidly formed and,

equally, a more readily decomposed material, *i.e.* the cells of the micro-organisms, will play the deciding role in the supply of nitrogen to the plant community, either through the metabolic products excreted or by the decomposition of the microbial cells.

It has been established that in cultures of Azotobacter 10 to 25 per cent of the nitrogen present is extracellular (Burk and Horner [7], Roberg [41]): moreover, that in mixed cultures a greater efficiency of fixation may occur (*cf* Roberg). This would support the contention that the nitrogen-fixing bacteria initiate the process of microbial cell multiplication in the phyllosphere, with a follow-up of a number of other micro-organisms living on the excreted matter, and on the living or decomposing cells. Moreover, these products may be easily absorbed by the green plant. The constancy of the weight of the living leaves in an established vegetation appears to be significant in this connection since the foliage is the production centre of the organic material to be used on one side as plant-building substance or on the other side as the energy source of the phyllosphere population.

Summarizing it may be said that bacteria able to fix atmospheric nitrogen abound in the soil and in the phyllosphere; under suitable environmental conditions these organisms contribute to the supply of combined nitrogen for all the other organisms of the habitat. In the soil fully adequate conditions occur in root nodules. The close association of the two symbionts in these structures allows direct exchange of metabolic products. The role of the free-living nitrogen-fixing bacteria of the soil, however, is still in doubt and might be partly ascribed to the lack of an adequate supply of suitable energetic material. Increasing attention is being given to the rhizosphere population living on root exudates, apart from the bacteria living on the decomposition products of dead plant material, which as has been mentioned before are released in small amounts during short flushes after rewetting of the soil. It seems possible, however, that the big gains under grass leys as reported by Parker may be a joint effect due to the sugars exuded by the leaves and washed into the soil. In both these cases the higher plant is the ultimate source of energy material for the micro-organisms concerned. On the other hand in nitrogen-deficient soils the growth of micro-organisms and their nitrogen-fixing activities are recognized as essential for the survival of the plant cover, particularly so in the first stages of recolonization of bare soil.

A close association between micro-organisms and green plants also occurs in the phyllosphere. Here it is the transpiring leaf which supplies the necessary organic and mineral nutrients for the develop-

ment of the phyllosphere population. Free water as a major factor in this environment enables the phyllosphere population to use the leaf exudates and allows an exchange of materials to take place between the leaf and the phyllosphere. In this exchange proximity to the site of photosynthetic activity may prove to be highly advantageous. The diurnal rhythm of the variation in water supply controls excretion and release as well as absorption of materials. Variations in wetting, moreover, induce intermittent development and recession of part of the phyllosphere population during the wet and the dry periods respectively. From the intermittent flow of nutrients to and from the leaf, as well as from the phyllosphere, a steady nutritional state may result, which only gradually changes with the age of the leaf and the alterations in the composition of the microbial population. The readily decomposing microbial bodies provide directly available nutrients for both the higher plant and the phyllosphere population in short, daily flushes. The role of the free-living, nitrogen-fixing bacteria in this environment is likely to be of more consequence here than in the soil.

The discussed interrelations are schematically arranged in Fig. 3.

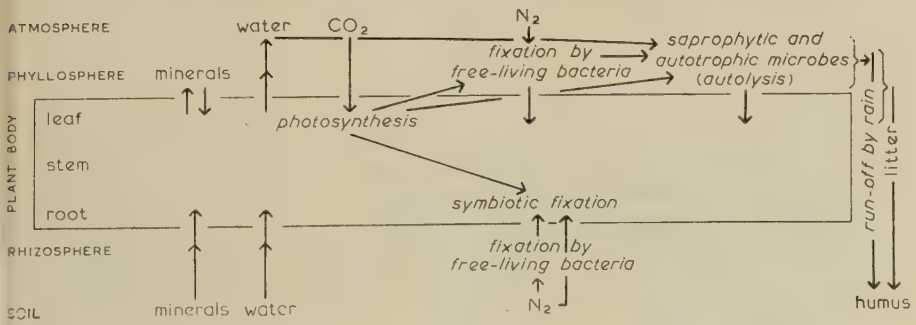

Fig. 3. Explanation in text.

It is obvious that the climate as a whole determines the existence of the phyllosphere population. The necessary conditions reach optimum levels in broad-leaf, evergreen forest of the moist tropics. The temperature is, generally, not a limiting factor. High relative humidity and daily wetting of the leaves either by rain, dew or condensed water vapour from the soil alternate with daily short periods of surface drying. The importance of these daily variations for the life of the higher plant and the phyllosphere population as

well as for the exchange of materials between the two has been emphasized. The production of organic matter by the vegetation is thus dependent on the supply of water.

This contention may account for the beneficial effect of plant cover on soil fertility and the rapid growth of tropical forest when once established, and also, for the relatively short periods of forest fallow allowable between two crops, as practised in shifting cultivation in the tropics. It might also be one of the reasons for the cultivation of tea, coffee and other tree crops, particularly in the early stages, under the shade of fast growing, albeit generally but not necessarily leguminous trees and also, for leaving the soil undisturbed and return to forest conditions, as advised by Harler [21] for the management of tea plantations. Eventually, it is in agreement with Willimott and Anthony's [53] suggestion for the South Sudan "that tree-crops in some form or other are the answer to the soil fertility–crop production problem".

Much research is needed to substantiate the above mentioned suggestions. They may, however, offer an alternative approach to the problems mentioned and open up a wider field of coordinated research in agriculture and forestry in the humid tropics.

SUMMARY

In the humid tropics a population of micro-organisms covers the foliage of the vegetation. It forms a continuous layer on the mature leaves and shows considerable variation in thickness and species composition on the upper and lower surface. A gradual change in distribution over the leaf surface and in species composition occurs during the physiological ageing of the leaves. It reaches a climax on the senescent foliage. By analogy with the rhizosphere, the term 'phyllosphere' has been applied to the micro-environment conditioned by the leaf.

Oligonitrophilic and nitrogen-fixing organisms abound in the phyllosphere. Among these, the representatives of the genera *Beijerinckia*, *Azotobacter*, *Aerobacter*, *Pseudomonas*, and *Spirillum* could be identified. They are the first organisms to colonize the leaf surface. The release of metabolic products from living and dying cells produces conditions which allow a succession of other organisms to develop.

The leaves retain a healthy green colour even under a considerable layer of micro-organisms unless unfavourable climatic conditions induce the ever-present semi-pathogens to infect the plant.

The phyllosphere as an environment is subject to influences from the whole plant as well as from the atmosphere, the changing population con-

tinually conditions it further. Climatic factors act directly as well as indirectly through the responses of the higher plant. Diurnal variations between physiologically considerable extremes are characteristic of the environment. Free water is essential for the phyllosphere organisms which consequently develop during the periods of wetting. Furthermore, water is the medium of exchange between the leaf and the phyllosphere population. Much depends upon the form in which the water is available, *i.e.* as rain or as dew. The duration of the wetting and the quantity of water determine the possibilities for development and survival of specific organisms in the wetting and drying cycle and, therefore, ultimately the species composition of the phyllosphere population.

The possible effects of this epiphyllic population on the growth of higher plants are discussed.

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NITRATE DISTRIBUTION IN TROPICAL SOILS

I. POSSIBLE CAUSES OF NITRATE ACCUMULATION NEAR THE SURFACE AFTER A LONG DRY PERIOD

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INTRODUCTION

Many investigators in the tropics report high nitrate content in the topsoil during the drier part of the year (Schofield [9], Hardy [3], Meiklejohn [6], Jones [4]). This phenomenon is not as yet understood, since soil temperatures are generally believed to be too high and soil water content too low during such periods for biological processes to take place.

This has been confirmed in laboratory studies by Meiklejohn [6] in which maximum temperatures for nitrification were lower than found in the field at the time and place of accumulation. According to Meiklejohn [6], there is some circumstantial evidence from field experiments in Kawanda, Uganda, for support of the photo-chemical oxidation hypothesis, as suggested by Dhar *et al.*[2]. *Viz* (i) the greatest accumulation took place in bare fallow soil exposed to sunlight, (ii) nearly all nitrate was in the top inch of the soil, and (iii) maximum temperatures were regarded as being too high and soil water content too low for bacterial activity.

This phenomenon of increase in nitrate content in the top soil during the dry season has been observed at the Katherine Research Station, Northern Territory, Australia, on bare fallow plots of Tippera clay loam. These plots are a part of an experiment in which certain aspects of the nitrogen cycle are being studied. The soil, derived from limestone, is a sandy clay loam overlying clay subsoils, with a pH of 6.5 in the topsoil (Stewart [11]). At 8 inches depth

the water content of this soil at 15 atmospheres is 12 per cent and field capacity 18 per cent (Slatyer, unpublished data).

The climate at Katherine has been described in some detail by Slatyer [10]. In the summary of his paper he states: –

"Geographically, Katherine is located at 132.3°E longitude, 14.3°S latitude and an altitude of 355 ft. Its climate falls into the broad description of tropical savanna and each year is divided into two major seasons – a warm dry winter period from May to September and a hot summer period from October to April. Virtually all the rainfall is received within the hot summer season, and most of it in the four-month period from December to March. The mean annual rainfall of 36.45 in. is received on an average of 59 wet days and is subject to a coefficient of variability of approximately 20 per cent, (100 S.D./mean).... The temperature regime is characterized by high temperatures throughout the year. Mean monthly maxima range from 85–90°F in the coolest months of June and July to 100–105°F in the hottest months of October and November. Minima likewise range from 50–55°F to 75–80°F. Very little variation occurs from year to year."

Experiments were carried out in order to reach a better understanding of the occurrence of high nitrate content in the surface of the soil during drying cycles.

EXPERIMENTAL

(*a*) *Experiment 1*

It was found at Katherine that soil nitrate content increases towards the surface when sampled in 1-inch layers at the end of a long dry period. If the actual cause of this distribution of nitrate was a phenomenon occurring at the surface of the soil, then it could be expected that a similar increase towards the surface would be revealed by sampling the soil in thinner layers. This was tested in Experiment 1.

The layout of the experiment consisted of three blocks, each including two sets of fallows. Both sets had been ploughed to 4 inches deep at the beginning of the wet season (December, 1956) and harrowed. Thereafter one set was left undisturbed, while the second set received two more cultivations in January and February, after which it was left undisturbed. On both sets of fallows weeds were controlled manually, avoiding disturbance of the surface of the soil as much as possible. As the bare fallows had an exceptionally

flat surface, they presented a good opportunity to measure nitrate content in thin layers in the top 3 inches.

On October 1, 1957 six and a half months after the last rain, samples were collected in each $\frac{1}{4}$-inch layer from the surface down to 3 inches on both sets of fallows. The sampling instrument consisted of a piece of wood from which tubes of $\frac{1}{4}$-inch internal diameter projected horizontally. One tube occupied each $\frac{1}{4}$-inch horizontal layer of the wood so that 12 tubes could simultaneously sample the top 3 inches of the soil. (The tubes were randomized in lateral distance along the piece of wood so that they were not located vertically over one another). In the actual sampling operation a vertical face was cut in the soil with a spade and the sampling instrument driven horizontally into the exposed face. Vertical motion was eliminated by steel guides which rested on the soil surface.

In this way 0.3 to 0.5 g of soil per sampling per layer was collected. The top $\frac{1}{4}$ inch of soil was taken separately with a small trowel. In each fallow plot eight samples per layer were taken, bulked, and throughly mixed. Nitrate was determined spectrophotometrically on 0.5-g subsamples, using phenoldisulphonic acid according to the method of Steinbergs (personal communication). The remainder of the samples from each layer was bulked, and Kjeldahl nitrogen, not including nitrate, was determined in triplicate on 3-g subsamples, using mercury and potassium sulphate as catalysts. Preliminary tests of this method showed that additions of nitrate did not significantly increase the nitrogen eventually distilled over as ammonia.

There was no statistical difference between the two fallow treatments, and in Figure 1 the mean of the absolute and log nitrate concentrations in ppm of oven-dry soil of both types of fallow

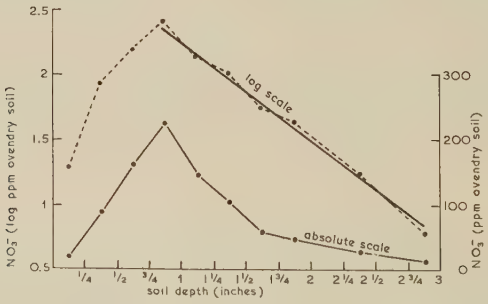

Fig. 1. Nitrate stratification in the top 3 inches of bare fallow soil after a $6\frac{1}{2}$ month rainless period, October 1947. (Nitrate contents are plotted on absolute and logarithmic scale).

for each layer are plotted against soil depth. The standard errors of the log concentration for each sampling depth are given in Table 1

TABLE 1

Standard error of the log of ppm nitrate values for each sampling depth in Experiment 1	
Sampling depth (inch)	Standard error (NO_3^-, log ppm)
$0-\frac{1}{4}$	0.063
$\frac{1}{4}-\frac{1}{2}$	0.025
$\frac{1}{2}-\frac{3}{4}$	0.082
$\frac{3}{4}-1$	0.039
$1-1\frac{1}{4}$	0.029
$1\frac{1}{4}-1\frac{1}{2}$	0.029
$1\frac{1}{2}-1\frac{3}{4}$	0.106
$1\frac{3}{4}-2$	0.134
$2\frac{1}{4}-2\frac{1}{2}$	0.303
$2\frac{3}{4}-3$	0.165

As the ten points are correlated, fluctuations in the curve cannot be evaluated by reference to these standard errors. However, the level of error indicates that the differences between the 0- to $\frac{1}{4}$-inch and $\frac{3}{4}$- to 1-inch layers, and again between the $\frac{3}{4}$- to 1-inch and the $2\frac{3}{4}$- to 3-inch layers, are highly significant. Figure 1 suggests an approximate linear relationship between nitrate concentration and depth in the surface to 1-inch layer, and a linear relationship between log nitrate concentration and depth in the 1- to 3-inch layer.

Table 2 shows that there was no significant difference in Kjeldahl nitrogen between the top four $\frac{1}{4}$-inch layers of the soil.

TABLE 2

Distribution of Kjeldahl nitrogen over the top inch of soil (Kjeldahl-N does not include NO_3-N)	
Soil depth (inches)	Kjeldahl-N (ppm)
$0-\frac{1}{4}$	546
$\frac{1}{4}-\frac{1}{2}$	569
$\frac{1}{2}-\frac{3}{4}$	553
$\frac{3}{4}-1$	562
S.E.	± 22.5

(b) Experiment 2

This experiment was designed to follow the course of accumulation of nitrate in the topsoil during a drying period immediately preceded by a wet period.

Another area of the same soil type which had been kept bare fallow in the preceding wet season (1957–58) was divided into three subplots, each enclosed by a square metal frame 6 × 6 ft. In July 1958, three months after the last rain, the soil was wetted with 1 inch of stored rain water. Natural rainfall was simulated by applying the water through a spray nozzle. The soil was rolled twice immediately after the disappearance of surface water in order to obtain a flat and packed surface soil. Nitrate content of the soil was determined at 0 to 1, 1 to 2, 2 to 3, 3 to 6, 6 to 9, and 9 to 12 inch levels at intervals until the first rains of the following wet season. In addition, samples were taken from the 0–½ and ½–1 inch depths in two of the three plots at three different dates. Samples were collected with small tubes vertically inserted into the soil. The diameter of the sampling tubes decreased with increasing sampling depth. At each occassion the samples were collected between 10 a.m. and 12 noon.

Changes in nitrate and soil water content after wetting are shown in Figure 2. Over a period of 9½ weeks after wetting, nitrate content in the 0–1 and 1–2 inch layers increased steadily to approximately

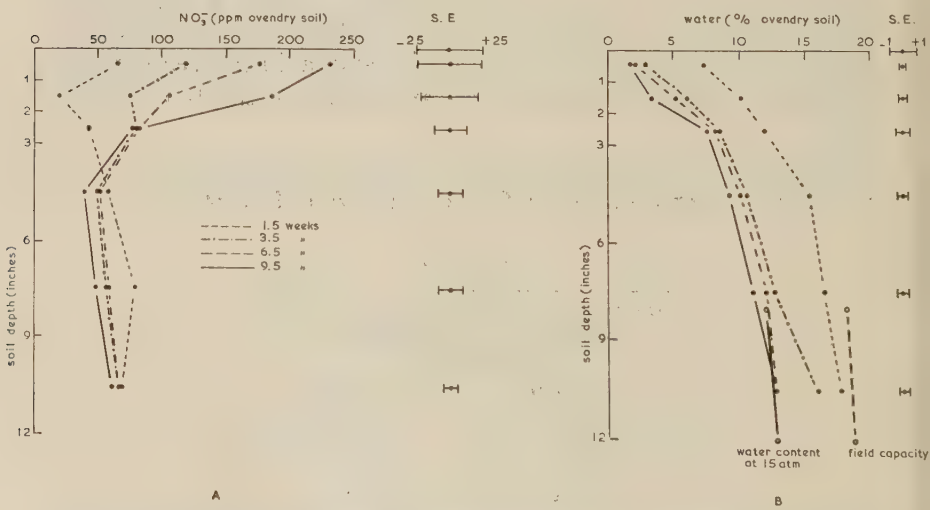

Fig. 2. Nitrate (*A*) and water (*B*) stratification in the top foot of bare fallow soil during the 1958 dry season, several weeks (as indicated) after artificial wetting of the soil.

200 ppm nitrate, the final concentration in both layers being significantly higher than that recorded after $1\frac{1}{2}$ weeks. Soil water content decreased rapidly in all layers during the first $3\frac{1}{2}$ weeks, after which the rate of drying was reduced. The changes in nitrate content in $\frac{1}{2}$-inch layers of the top inch of soil are given in Table 3.

TABLE 3

Nitrate concentration (ppm) for each $\frac{1}{2}$-inch layer in the top inch of soil after several weeks drought

Weeks after watering	Soil layer, inches	
	0–$\frac{1}{2}$	$\frac{1}{2}$–1
$1\frac{1}{2}$	110	15
$3\frac{1}{2}$	171	73
$6\frac{1}{2}$	208	114
S.E.	±13.2	±31.0

Both layers increased by about 100 ppm nitrate. The changes in the top $\frac{1}{2}$ inch were significant, those in the $\frac{1}{2}$-1 inch layer were not.

(*c*) *Experiment 3*

In this experiment the effect of solar radiation on changes in nitrate content was tested.

Soil from the top 2 inches of the fallow areas of Experiment 1 was collected at the end of the 1957 wet season, air dried, ground, and sieved. Sievings smaller than 0.5 mm were exposed to solar radiation in shallow metal trays of 3 inch diameter for different periods. Since light has a limited depth of penetration into the soil, different soil thicknesses ($\frac{1}{8}$–1 inch) were exposed, the depth of the trays corresponding to the thickness of the layers. In order to evaluate the effect of the shorter wavelengths, similar layers were covered with a thin sheet of tinplate, on the outside of which a soil surface was simulated by spreading a finely powdered soil onto the glue-painted outside surface of the tin. Contact between the covering sheet and the soil layer was ensured by filling up the tray such that the resulting soil surface was slightly convex. The cover was then pressed on to the surface in a circular motion. The trays were embedded into a 3-inch soil layer so that $\frac{1}{8}$ inch of the top of each tray was above the surface. The trays were kept outside continuously, and were covered with a sheet of plastic at times of rain.

In this way eight thicknesses of soil, from $\frac{1}{8}$ to 1 inch were exposed to solar radiation, covered and uncovered, for 2, 6, and 18 weeks. All treatments were duplicated. The lay-out was fully randomized.

The results are given in Figure 3, in which the average nitrate content for all thicknesses, covered and uncovered, is plotted

against hours of sunshine. Neither the differences between thicknesses of the layers nor the difference between the covered and uncovered soils were significant. For all thicknesses, covered and uncovered, nitrate content decreased with increasing hours of

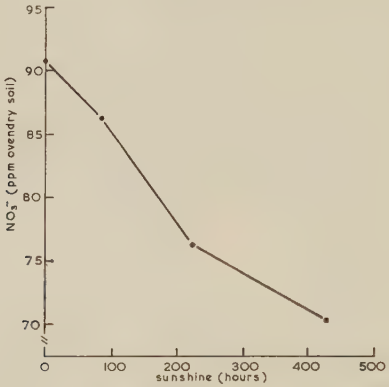

Fig. 3. Average effect of solar radiation on nitrate content after exposing different thicknesses of air-dray soil ($\frac{1}{8}$ to 1 inch) to all wavelengths or to the longer ones only.

exposure to solar radiation. Differences between dates of sampling were highly significant ($P = 0.001$), with a standard error of $\pm$ 1.46 ppm. The average decrease was about 0.05 ppm per hour of sunshine. Daily maximum soil temperature, measured at $\frac{1}{2}$ inch depth in a 1 inch thick layer, averaged 66.3° for the covered and 70.0°C for the uncovered soils.

DISCUSSION

In both experiments 1 and 2 the highest amount of nitrate in the top 3 inch was located in the top inch of soil (Table 4). This

TABLE 4

Nitrate concentration (ppm) per inch soil layer in top 3 inches after prolonged drought		
Soil depth (inches)	Expt. no	
	1	2
0–1	123	232
1–2	86	187
2–3	17	78

confirms the findings of Mills [5] in Uganda, who found the highest nitrate content in the top inch (300 ppm), with a sharp drop to 40–60 ppm in the second inch. These figures could be construed as support for the possible occurrence of photochemical oxidation, since in 1-inch layers nitrate content appears to increase towards the surface. However, the more detailed sampling of Experiment 1 revealed that there is a marked decrease from $\frac{3}{4}$–1 inch depth towards the surface. According to Baumgartner [1] only about 10 per cent of the light falling on the surface of a soil reaches 1/10 inch depth when the soil has a grain size of 1 to 0.5 mm, the shorter wavelengths being absorbed most. Thus it is very unlikely that ultraviolet light affects the soil to a depth of $\frac{3}{4}$ inch. Even if it did, its effects would presumably diminish from the surface down to this level, whereas Experiment 1 showed an increase in soil nitrate over this range. It could be reasoned that nitrate is formed photochemically at the surface, but diffuses downward. According to Stewart and Eck [12], surface-applied nitrate moves downwards to $1\frac{1}{2}$ inch when the soil is at 8–15 atm soil-water tension. The distribution of nitrate content they obtained after movement was a strict logarithmic decline from the surface to that depth. Figure 1, on the other hand, shows a linear increase from the surface down to $\frac{3}{4}$ in. Moreover, in Experiment 3, exposure to solar radiation of thin air-dry layers of soil actually brought about a steady decrease in soil nitrate with time. The fact that this decrease took place in covered as well as in uncovered trays indicates that the heat component of the radiation is the actual cause of the decrease in nitrate content. It might be argued that the extractability of nitrate may decrease with temperature. However Steinbergs (personal communication) has shown that this is not the case with the method used by the author.

It is evident from the above that the high nitrate content in the top inch of the soil is not likely to be due to photochemical or physico-chemical oxidation. It is also clear that nitrate formed in any other way at the actual surface could not account for the distribution of nitrate with depth shown in Figure 1.

If it is assumed that nitrate is formed biologically, then the distribution of nitrate between the surface and 1 inch depth (Figure 1) should be explicable in terms of soil water and temperature effects. According to Meiklejohn [7], cultures of nitrifying bacteria, de-

rived from an arable soil in Uganda, did not nitrify above 40°C. Robinson [8] found that in a Kenya red-loam coffee soil nitrification stops at a soil water content just below permanent-wilting percentage. In Experiment 1, in which nitrate accumulation in the top two inches took place during a drying cycle, without any interference from rain, conditions favourable for biological nitrate formation would have been maintained for a longer period at greater depth. Figure 1 would seem to confirm this, as nitrate content increases from the surface towards 1 inch depth. However, it does not explain the decrease in nitrate content below the $\frac{3}{4}$–1 inch layer, since soil temperatures and soil water levels would be more favourable for nitrification in that zone.

Experiment 2 shows that water contents between 1 and 3 inches might be adequate for biological nitrification for $3\frac{1}{2}$ weeks after the commencement of the drying period.

After that period, however, nitrate content continued to increase in the 0–1 and 1–2 inch layers, as can be seen in Figure 2. It is possible that during the night, due to temperature gradients, water condensed towards the top soil to raise the water content to a level high enough for bacteria to nitrify, particularly as temperatures would then have been well below the critical value of 40°C. However, in Experiment 2 nitrate content increased during a period in which water content at mid-day was between 1.6 and 1.9 per cent, and it is most unlikely that water content would have been increased sufficiently by night condensation to reach a level to permit biological nitrification.

Table 2 does not reveal any marked difference in Kjeldahl nitrogen between the top four $\frac{1}{4}$-inch layers. Though a decrease in Kjeldahl nitrogen, due to local nitrification, might have been possible within the limits of the standard error, there does not seem any strong indication for it. Furthermore it has been found (Wetselaar, in preparation) that, on the same soil type, surface-applied sulphate of ammonia on fallow plots nitrified very slowly during the wet season, and nitrification ceased altogether during a two-week dry spell. This would indicate that water shortage restricts nitrification fairly rapidly. This restriction would certainly be operative during the latter half of the drying period in Experiment 2.

Since the phenomenon of nitrate accumulation cannot be explained in terms of photochemical, physico-chemical or biological

processes, it would seem logical to investigate the possible physical aspects involved. Biological formation of nitrate will probably proceed in the soil as long as the soil water conditions will allow. According to Figure 2B this may have lasted for $3\frac{1}{2}$ weeks after wetting (Experiment 2) below 2 in. depth. During this period, and continuing to at least $9\frac{1}{2}$ weeks after wetting, upward capillary movement of water may have taken place. Locally formed nitrate could thus have been moved upwards with the water and could have accumulated near the surface where conditions for biological formation were unfavourable.

Supporting this theory is the fact that in Experiment 2, in which the soil had been compacted after wetting, the nitrate in the top $\frac{1}{2}$ inch increased as fast as in the lower $\frac{1}{2}$ inch. Thus compared with Experiment 1 packing of the topsoil gave an increase in nitrate content to a shallower depth than in the non-packed soil.

Further investigations of upward movement of nitrate are presented in Part II of this series of papers (Wetselaar [13]).

SUMMARY

On a lateritic red earth in Northern Australia nitrate accumulates in the top inch of the soil during the dry season (April-October). Detailed measurements in the top 3 inch of the soil in $\frac{1}{4}$-inch layers revealed that the actual peak of accumulation is not at the surface but at $\frac{3}{4}$–1 inch depth. Above this zone nitrate content decreases linearly towards the surface, below it nitrate content decreases logarithmically towards 3 inches depth. In view of the shallow penetration of ultraviolet light into the soil the high nitrate content cannot be explained by photochemical oxidation. This has been confirmed by exposing soil layers of different thicknesses, covered and uncovered, to solar radiation. The resultant significant decrease in nitrate content with time points to a reverse effect caused by the heat component of the radiation.

In another experiment the same soil type was artificially wetted once during the dry season, and changes in nitrate content were followed in different layers in the top foot of the soil during a continuous drying cycle. Nitrate accumulated in the top 0–1 and 1–2 inch zones and tended to decrease in the 6–9 and 9–12 inch zones during the $9\frac{1}{2}$ week drying period. Packing of the surface soil after wetting resulted in a location of maximum nitrate content in the top $\frac{1}{2}$ inch of the soil.

The results cannot be explained in chemical or biological terms. A physical movement of nitrate is suspected.

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NITRATE DISTRIBUTION IN TROPICAL SOILS

II. EXTENT OF CAPILLARY ACCUMULATION OF NITRATE DURING A LONG DRY PERIOD

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INTRODUCTION

Accumulation of nitrate near the surface of a bare fallow soil at Katherine, N.T., was described in the first of this series of papers (Wetselaar [5]). It was shown that neither by photo-chemical, physico-chemical, nor by biological formation could this phenomenon be explained. Accumulation of nitrate by physical means was suggested.

This paper reports investigations on three soil types in the Katherine region – Tippera, Blain and Florina soils. Tippera is a lateritic red earth which has been described in Part I of this series (Wetselaar [5]). Florina is a sandy lateritic podzolic soil (Stewart[3]), Blain a sandy lateritic red earth (Stewart, personal communication), both with a clay content of about 5 per cent in the top soil.

EXPERIMENT 1

The changes in nitrate content during the dry season were compared with those of an anion of similar solubility that is not affected biologically. The chloride ion was chosen for this purpose.

At the beginning of the wet season, December 1958, sodium chloride at the rate of 2000 pounds per acre was hand broadcasted on triplicate plots, 4 × 12 feet on each soil type. Immediately adjacent to these plots (the "chloride plots") were triplicate plots, 8 × 12 feet, receiving no salt (the "nitrate plots"). In order to avoid chloride contamination from the NaCl

application during broadcasting and subsequent possible horizontal diffusion in the soil, chloride and nitrate plots were not randomized among each other.

The cultivation history for all plots and for all soil types was exactly the same. Each had been bare fallow for one year, after clearing from virgin land the previous season. On 1st December, 1958, all plots were ploughed to 8 inches, and then harrowed. Two days later NaCl was applied to the chloride plots. No further cultivation took place during the wet season. Weeds were controlled manually.

Three samples per plot were collected for chloride and nitrate on the respective plots during the dry season from the 0–1, 1–2, 2–3, 3–6, 6–9, and 9–12 inch soil levels, using tubes diminishing in diameter with increasing depth. For each plot the samples were bulked, mixed, and sub-sampled in the field. On each sampling date the Florina samples were collected between 8 and 9, the Blain samples between 9 and 10, and the Tippera samples between 10 and 11 a.m. Extraction for soil nitrate took place within 4 hours after sampling on 10–g subsamples. Nitrate was estimated by the method described in Part I of the series (Wetselaar [5]). The rest of the subsample was used to determine soil water content gravimetrically. The chloride subsamples were first oven dried at 105°C to determine soil water content. The dry soil was then ground and mixed, 20 ml distilled water was added to a 10-g subsample, followed by shaking for one hour. Chloride concentration was estimated in the supernatant, using a modification of the method of Stern and Schwachman [4].

The first sampling took place on 21st April, when it was expected that the rains for the season would have finished. However, on 18th May Tippera received 0.08 inch rain, and on 24th May Tippera received 0.85 inch, Blain 0.51 inch, and Florina 0.54 inch. The final sampling was on 21st July.

Disturbance of the soil crust during sampling was avoided by working from planks which rested on the soil on the edges of the plots.

The sampling holes were filled up again with the residue of the bulked samples after sub-sampling. The holes were then marked to avoid re-sampling in the same position.

Changes in nitrate, chloride and soil water content between 21st April and 21st July on Tippera, Florina, and Blain soils are shown in Figures 1, 2, and 3 respectively. For Blain, chloride content and soil water content on chloride plots are omitted, since it was found that practically all chloride applied had been leached out of the top two feet of this soil during the wet season. The residual chloride fluctuated between 6 and 10 ppm at all sampling depths. This difference was too small to evaluate trends of accumulation at any depth.

The sampling period covered three phases, a drying phase from 21st April until 13th May, a rainy period from 13th May to 28th

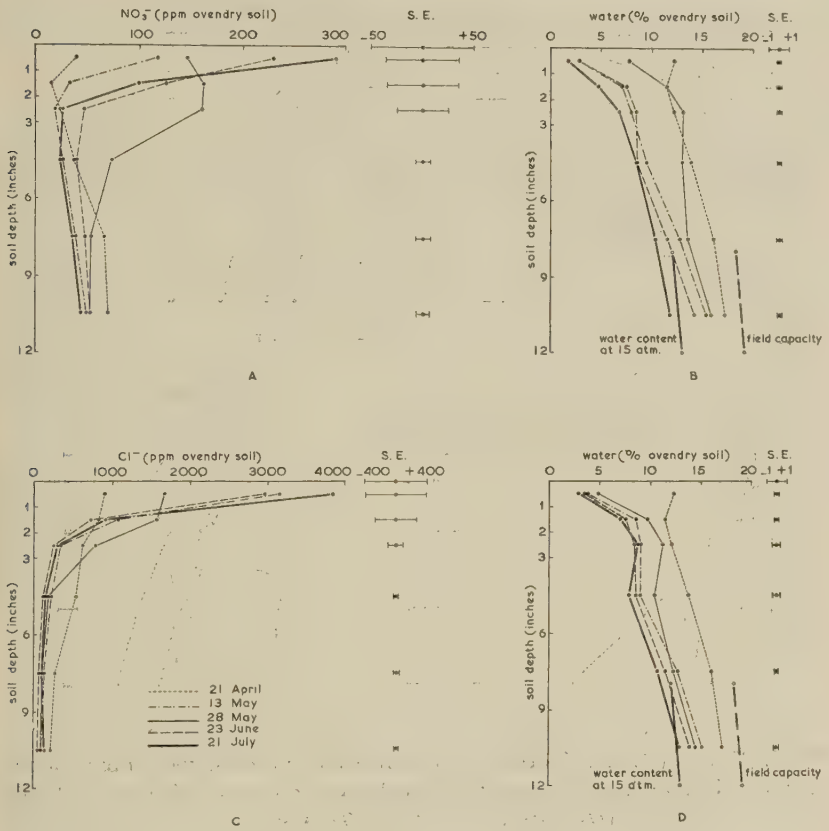

Fig. 1. Nitrate (*A*) and chloride (*C*) stratification in the top foot of bare fallow Tipera clay loam, together with changes in soil water on nitrate (*B*) and chloride (*D*) plots, during the 1959 dry season.

May, and a second drying phase from 28th May to 21st July. The Tippera soil dried to approximately the water content at 15 atm to 9 inches by 13th May. The May rain wetted the soil to 6 inches. This water was lost through evaporation by 23rd June, when the water content profile approached that of 13th May. By 21st July the whole 12-inch profile was below the 15-atm level. Florina and Blain showed similar cycles, but data on 15-atm level and field capacity are not yet available for these soils.

On Tippera soil, changes in nitrate and chloride content with time were significant in the 0–1, 2–3, and 3–6-inch zones, and changes in chloride were also significant in the 9–12-inch zone (Figures 1A

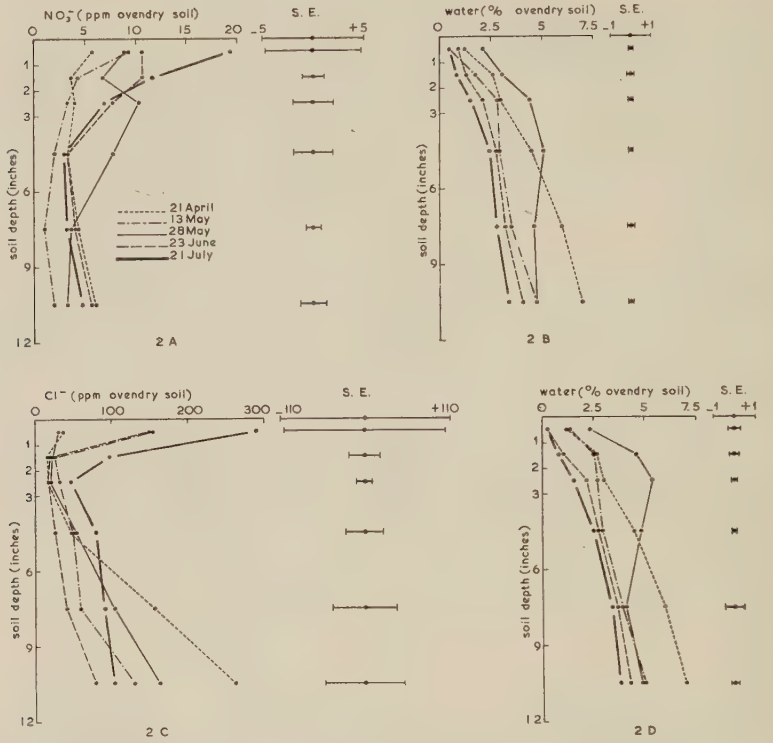

Fig. 2. Nitrate (*A*) and chloride (*C*) stratification in the top foot of bare fallow Florina soil, together with changes in soil water on nitrate (*B*) and chloride (*D*) plots, during the 1959 dry season.

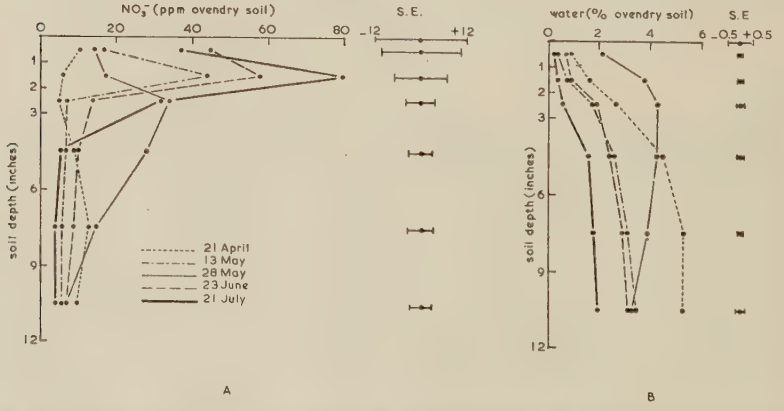

Fig. 3. Nitrate (*A*) and soil water (*B*) stratification in the top foot of bare fallow Blain soil, during the 1959 dry season.

and 1C). Significant changes and trends for the drying phases and the rainy period are shown in Tables 1 and 2 respectively.

TABLE 1

Effect of drying cycles (21.IV-13.V 1958 and 28.V-21.VIII 1958) on changes in nitrate and chloride content in each sampling layer on each soil type. Table shows depth(s) of soil layers() in inches where a change has been observed as indicated in the first column

Changes in	Chloride				Nitrate					
Soil type	Tippera		Florina		Tippera		Florina		Blain	
Drying phase . . .	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Significant * increase	0–1	0–1				0–1		1–2	1–2	1–2
Trend towards increase	1–2		0–3	0–6	0–2		0–2	0–1	0–1 2–3	0–1
Trend towards decrease	6–9	1–2 3–6	6–12	9–12	2–12	1–2 6–12	2–12	2–3	3–12	6–12
Significant * decrease	2–6 9–12	2–3				2–6		3–6		3–6

* $P = 0.05$

TABLE 2

Effect of rain between 13.V and 28.V 1958 on changes in nitrate and chloride content in each sampling layer (depth in inches) on each soil type

Changes in	Chloride		Nitrate		
Soil type	Tippera	Florina	Tippera	Florina	Blain
Significant * increase	2–3		2–6	2–6	2–6
Trend towards increase	1–2	3–12	0–2 6–12	1–2 6–12	6–12
Trend towards decrease		0–1 2–3			0–1
Significant * decrease	0–1				1–2

* $P = 0.05$

During the first drying phase, nitrate and chloride content on Tippera increased in the top 2 inches and decreased in the lower layers. Following the May rain, chloride decreased in the top 1 inch and increased in the 1–3-inch layer, but nitrate content tended to increase throughout the profile.In the second drying phase, both nitrate and chloride again increased in the top 1 inch and decreased in the lower layers. The pattern of change on Florina soil was very similar to that on Tippera (Figures 2A and 2C).

On Blain soil (Figure 3A) gross changes in nitrate stratification during the drying phases were similar to those on Tippera and Florina, but in this instance the zone of maximum nitrate accumulation was at 1–2 inches depth.

Discussion

From the results it is clear that the general effect of a drying phase is a marked accumulation of both chloride and nitrate anions near the surface, accompanied by a decrease in the subsurface.

The reverse effect, surface depletion and subsurface accumulation caused by rain, is clearly shown with chloride, but does not fully hold for nitrate, since on Tippera and Florina there was no decrease in nitrate in the top inch of the soil after wetting. However the fact that the increase in subsurface anion concentration was much greater for nitrate than for chloride indicates that some nitrate was formed locally during the wet period on these soils when water content was adequate. Though the changes in total amount of nitrate and chloride with time in the top 12 inches, given in Table 3, were not significant, total nitrate content was nearly doubled on all three soil types during the wet period between 13th May and 28th May.

TABLE 3

Changes in total nitrate and chloride contents (ppm) in top 12 inches

	Nitrate			Chloride	
Soil	Tippera	Blain	Florina	Tippera	Florina
Date					
21.IV (dry)	194	38	18	1838	1465
wet 13.V (dry)	166	41	11	1959	912
wet 28.V	312	70	23	1725	1022
23.VI (dry)	268	63	26	1559	622
21.VII (dry)	229	63	24	2118	1252
S.E	33.7	9.1	3.4	232.6	125.7
	n.s.	n.s.	n.s.	n.s.	n.s.

The effect of rain in redistributing anions can be ascribed to leaching even if nitrification during the rainy period is taken into account, since changes in stratification of the chloride ion, which is not subject to biological action, were parallel to those of the nitrate ion (Figures 1A and 1C). Both the chloride and nitrate

stratifications at 28th May are in agreement with the results of other experiments (Wetselaar in preparation), in which parallel distribution of these anions with depth was recorded.

During the drying phases, total nitrate content over the 0–12 in. profile did not increase (Table 3), indicating that biological nitrate formation ceases altogether under these soil temperature and water conditions. On Tippera soil there was a tendency for the total nitrate content to decrease. A significant reduction in total nitrate content was shown under laboratory conditions in dry Tippera soil exposed to high temperatures (Wetselaar [5]).

Detailed triplicate soil temperature measurements at the surface and at $\frac{1}{2}$, 1, $1\frac{1}{2}$, 2, 3, and 6 inches depth, using thermistors, revealed that during the sampling period maximum temperatures at $1\frac{1}{2}$ inch or below never exceeded 40°C. Assuming that biological nitrate formation can continue up to this temperature, it is clear that the cessation of biological activity was due to low soil water content. However, during these drying phases, nitrate content increased in the surface and decreased in the subsurface layers. Thus it appears that redistribution of nitrate takes place during a period in which water content levels are too low for biological nitrate formation, and during which no change in total anion content took place. This and the fact that changes in chloride stratification can parallel to those of nitrate suggest a common physical cause.

The accumulation of chloride and nitrate near the surface during drying phases can therefore only be explained in terms of capillary movement upwards of liquid water plus solute along a marked moisture gradient towards the surface, with a consequent decrease in anion content in the subsurface. Marshall [1], reviewing the literature on upward movement of salts, states that water can move in the liquid phase under very much drier conditions than was originally assumed. Marshall and Gurr [2] obtained a shift of 50 per cent of the chlorides, originally in the bottom 1 cm, towards the top cm in a 2-cm block of soil at a water content only slightly above the 15-atm level. The shift took place within 24 hours. The results presented in this paper confirm this, and seem to indicate that even below the 15-atm level some liquid movement upwards can occur. It can be concluded therefore that the main cause of nitrate accumulation near the surface during a long dry period is capillary movement upward of the nitrate formed in the subsurface.

Since there is no apparent increase in total chloride content in the top foot during a drying phase (Table 3), all increases in chloride content near the surface can be accounted for as changes within the top foot of soil. Nitrate measurements in the field at the end of the dry season on a fallow soil high in nitrate content invariably revealed a marked decline to near zero at about one foot depth and a subsequent increase in the deeper layers (Wetselaar, unpublished data). Furthermore, on the same fallow soil, water content below the $1\frac{1}{2}$–2-ft soil layer showed scarcely any decrease during the dry season (Arndt, personal communication). Thus it can be concluded that, under Katherine conditions, the upwards movement of soil water in the liquid phase during the dry season is mainly restricted to the top $1\frac{1}{2}$ feet.

EXPERIMENT 2

From field observations it seemed that the zone of maximum nitrate accumulation was associated with the base of the soil crust, formed by raindrop puddling. This crust consists of an upper cemented layer, $\frac{1}{8}$–$\frac{1}{4}$ inch thick, overlying a layer that is looser and more open in structure and apparently lower in clay content. This texture differentiation is caused by differences in the speed of settling after rain puddling, between coarse and fine particles.

The aim of the experiment was to investigate any relation that might exist between location of physical discontinuity of the soil and location of maximum anion accumulation in the top 2 inches.

In addition to the nitrate plots on all soil types and the chloride plots on Tippera soil used in Experiment 1, which had not been cultivated since the ploughing and harrowing in the beginning of the preceding wet season, measurements for this experiment were also carried out on a block of three other chloride plots on Tippera soil, that had been harrowed regularly until one month before the end of the wet-season rains. In this way it became possible to make comparison between crust formation over a full wet season, and after the last rains only.

To measure the variation in physical continuity a specially designed penetrometer was used, which is described and illustrated (Figure 4) in the Appendix. The readings of this instrument represent the minimum force required to penetrate a soil face in horizontal direction, and are expressed in dynes cm^{-2}. Penetrometer readings were obtained in the top $\frac{1}{8}$ inch and in each $\frac{1}{4}$ inch to a depth varying from $1\frac{1}{2}$—$2\frac{1}{2}$ inches. Soil samples were collected in $\frac{1}{4}$-inch layers to a depth of $1\frac{3}{4}$ inch on the nitrate plots and on the unculti-

vated and cultivated chloride plots of the Tippera soil. The method of collection of these samples has been described by Wetselaar [5]. The averages given in Table 4 and 5 are based on three replicas per plot. Each replica penetrometer reading was again the average of two measurements. All readings and samples were taken on 17th August, that is 4 weeks after the last sampling in Experiment 1. No rain fell during this period.

The penetrometer readings on nitrate plots of the three soil types are given in Table 4, together with the readings on the different chloride plots on Tippera soil. No statistical comparison can be made between the different depths for one soil type. However, the

TABLE 4

Penetrometer readings in top 2½ inches, in nitrate and chloride plots, in dynes cm^{-2}

epth ches)	Nitrate plots			Chloride plots, Tippera	
	Blain	Florina	Tippera	Unharrowed	Harrowed
—⅛	*	*	>24000	4000 ± 1266	>24000
⅛—¼	1700 ± 444	*	9540 ± 3850	>24000	>24000
	460 ± 190	3080 ± 640	12600 ± 7090	>24000	22000 ± 2190
	760 ± 166	6140 ± 3054	>24000	>24000	4660 ± 730
	1300 ± 342	8800 ± 3726	>24000	>24000	>24000
	1140 ± 420	12460 ± 4086	>24000	>24000	>24000
	7900 ± 2824	14860 ± 2420	>24000	>24000	>24000
	13400 ± 4734	17340 ± 4444	>24000	>24000	>24000
	15360 ± 3716	>24000			
	17100 ± 2610	>24000			
	23000 ± 1000				

Loose sand, penetration resistance zero

measurements on the Blain soil indicate a marked change in penetrability below 1 inch depth, while on the nitrate plots of the Tippera soil penetrability decreased significantly between ¼ and ½ inch depth On Florina soil there was no sudden change at any depth.

The readings on the unharrowed chloride plots of the Tippera soil revealed a thin crust less than ⅛ inch thick, immediately underlain by a very hard soil. The harrowed chloride plots, however, had a thin hard surface about ¼ inch thick, underlaid by a loose zone between ½ and ¾ inch depth on top of hard soil.

Table 5 gives nitrate content on Tippera soil for the different top soil layers, as well as the chloride concentrations on the unharrowed and harrowed plots. There was a marked increase in nitrate content from the top ¼ inch towards ¾ inch depth, followed

by a decline towards $1\frac{3}{4}$ inch. The peak in nitrate content occurred in the $\frac{1}{2}$–$\frac{3}{4}$-inch layer.

TABLE 5

Nitrate and chloride contents (ppm), as on 17.VIII, on Tippera soil

Depth (in.)	Nitrate	Chloride	
		Unharrowed	Harrowed
0 –$\frac{1}{4}$	50 ± 35.4	4121 ± 607.2	2009 ± 497.0
$\frac{1}{4}$–$\frac{1}{2}$	251 ± 156.3	2961 ± 414.5	1068 ± 167.0
$\frac{1}{2}$–$\frac{3}{4}$	353 ± 179.5	2229 ± 554.7	3770 ± 2265.0
$\frac{3}{4}$–1	258 ± 8.2	1642 ± 190.7	3827 ± 760.0
1 –$1\frac{1}{4}$	82 ± 33.2	1039 ± 523.1	437 ± 174.0
$1\frac{1}{4}$–$1\frac{1}{2}$	36 ± 15.8	859 ± 436.8	89 ± 39.0
$1\frac{1}{2}$–$1\frac{3}{4}$	10 ± 2.6	230 ± 27.2	82 ± 11.0

Maximum chloride content was located in the top $\frac{1}{4}$ inch on the unharrowed plots and in the $\frac{3}{4}$–1-inch layer on the harrowed plots.

The coincidence between the zone of maximum anion accumulation and location of the base of the crust, as revealed by the penetrometer readings, is given in Table 6.

Discussion

When these soils are dry the non-puddled part sets hard, the clay fraction acting as a cement between the sand grains. The penetrometer readings can be considered as a measure of the degree of this cementation. Thus the softer, easier penetrable layer at the base of the crust has less clay cementation, *i.e.* less physical continuity, than the soil below it. This would result in a break in the continuity of a capillary water film containing solutes. Thus the base of the crust tends to become the interface between capillary movement below and vapour movement above it, with solutes concentrating at the bottom of, or just under, the interface.

The results, as given in Table 4 and 5, and particularly in Table 6, fully confirm this. Both nitrate and chloride are affected by the type and thickness of the crust. In case of nitrate this is clear from the difference in depth of the accumulation zone between Tippera and Blain soil (Table 6) and in the case of chloride in the difference between harrowed and unharrowed plots (Table 6). This again, points to a common cause of accumulation near the surface, for which again capillary movement is the only feasible explanation.

Apart from the capillary movement upward the anion would

tend to move downwards against the concentration gradient by diffusion. As was discussed in Part I of this series (Wetselaar [5]) downward diffusion results in a logarithmic decline in concentration

TABLE 6

Coincidence between zone of maximum anion accumulation and location of base of crust

	Tippera			Blain
	Nitrate plots	Chloride plots		Nitrate plots
		Unharrowed	Harrowed	
Base of crust revealed by penetrability, inches . .	$\frac{1}{2}$	$\frac{1}{8}$	$\frac{3}{4}$	$1\frac{1}{4}$
Zone of maximum accumulation of anion, inches	$\frac{1}{2}-\frac{3}{4}$	$0-\frac{1}{4}$	$\frac{3}{4}-1$	$1-2$

with increasing depth. In this way the logarithmic decline in nitrate content between $\frac{3}{4}$ and 3 inches, as given in Part I, is fully explicable, being the result of accumulation upward to the bottom of the crust and downward diffusion against the concentration gradient.

SUMMARY

Changes in nitrate and chloride content were followed in the 0–1, 1–2, 2–3, 3–6, 6–9- and 9–12 inch zones during the 1958–59 dry season on three different soil types at Katherine, N.T. Chloride was applied as NaCl to the soil surface at the beginning of the preceding wet season, which allowed most of it to leach towards the subsoil.

Generally, during drying phases nitrate and chloride increased in the surface layers and decreased in the sub-surface layers. The only rainy period resulted in a reverse effect. Both anions were affected similarly by weather conditions. In addition total nitrate tended to increase immediately after rain due to biological formation in the rewetted zone. Maximum accumulation of both anions during drying phases occurred in the 0–1 inch layers on two soil types and in the 1–2-inch layer on the third.

Penetrometer measurements revealed that the actual zone of accumulation is governed by the location of the bottom of the crust, which is easily penetrable.

It is concluded that the high nitrate content generally found in the surface of tropical soils after a dry period is due to capillary movement upwards of nitrate formed biologically in the subsurface during periods of adequate water content. The nitrate accumulates under the zone where the physical continuity of the soil is broken.

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APPENDIX

The apparatus (Fig. 4) with which changes in penetrability in a horizontal direction of the surface soil were measured consisted of an $1\frac{1}{2}$-inch wide plate A with a $\frac{1}{4}$-inch wide vertical slot down its centre, through which the 1/10-inch thick rod H of the penetrometer P could reach the soil, when the latter was moved in its bearing D horizontally towards the soil face. This

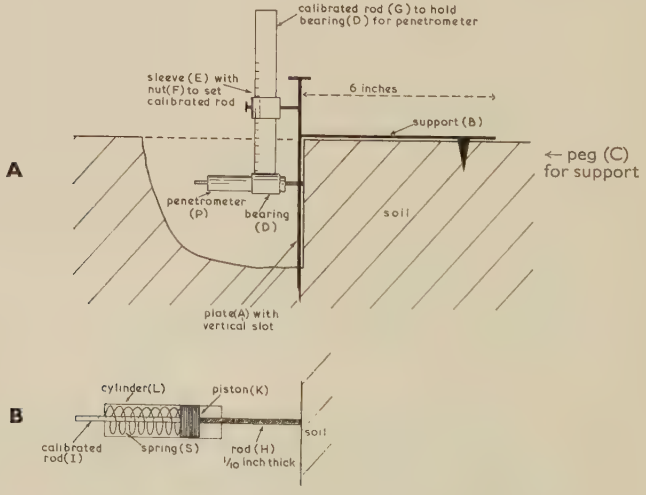

Fig. 4. Instrument as placed in the soil (A) for penetrometer readings. B is a longitudinal cross-section through penetrometer.

bearing was held by a calibrated rod *G* which could be set at the required depth by a nut *F* in a sleeve *E*. After carefully cutting away the top two inches of the soil with a small trowel the apparatus was hammered into the soil with the penetrometer at its highest position. The horizontal support *B* then rested parallel to and on the soil surface, secured with a peg *C*. The soil behind Plate *A* was then removed and the penetrometer lowered to the required depth. The penetrometer (Fig. 4B) consisted of a cylinder *L* in which the movement of a piston *K* away from the soil face was opposed by a spring *S*. Measurements of penetrability were taken by pressing the cylinder *L* towards the soil until the calibrated rod *I* would not extend any further. The calibration on rod *I* was then read. These calibrations were obtained by applying known pressure to 1/10-inch thick rod *H* in the laboratory for the different springs used. In many cases the soil was so hard that even with the heaviest springs penetration was not achieved even at maximum pressure. In these instances the readings are referred to as "higher than 24000."

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A UNIVERSAL METHOD FOR PREPARING NUTRIENT SOLUTIONS OF A CERTAIN DESIRED COMPOSITION

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INTRODUCTION

Water cultures, or other ways of soil-less plant culture, *e.g.* sand cultures, are frequently used for physiological research. Reference to the literature however, reveals a considerable diversity in the composition of the nutrient solutions used. Hewitt [2] gives over a hundred formulae, and Schropp [4] about sixty, excluding those prescribed for inducing deficiency symptoms. The development of practical soil-less culture techniques before the last war gave rise to a veritable avalanche of formulae. Up to the present time, a total of about three hundred formulae have been published, many of which have been particularly recommended for the growth of particular crops, *e.g.* tomatoes, carnations, roses *etc.*

Physiological experiments on nutrition have been made to investigate, among other things, the influence of a wide range of nutrient-solution composition on the development and behaviour of plants. For experiments of this kind, knowledge of the problems underlying the preparation of the desired nutrient solutions is a first prerequisite since the actual composition may not always correspond to that given by the formula. For example, there are many solutions, the formulae for which indicate the presence of much more phosphate, sulphate, calcium, and magnesium than can be determined by analysis; thus, from two solutions which, according to their formulae, should both contain say, 2 mg ions of phosphate per litre, 1.8 mg ions may be recovered from one, and only 0.2 mg ions from the other. This is due to the fact that the

great majority of recommended formulae are not realizable in solution because the solubility products of certain ion combinations are exceeded. The interpretation of experiments involving such cases would therefore, be seriously affected. The main object of the present investigation is to find a solution to these problems.

Although presenting no direct problems *per se*, it must also be noted that very divergent formulae may produce nutrient solutions of identical composition, *e.g.* 2 mmol Ca $(NO_3)_2$ and 4 mmol KH_2PO_4 give exactly the same concentrations of K^+ (4 me), Ca^{++} (4 me), NO_3' (4 me) and H_2PO_4' (4 me) as 4 mmol KNO_3 and 2 mmol $Ca(H_2PO_4)_2$: this is clearly seen when the ions are considered individually.

Judging from their publications and statements, it would seem that many investigators have grown crops in a solution chosen largely at random, and that, where the results have been successful, they have recommended that solution as being specific for the particular crop concerned. Comparisons between different solutions as to their effect on crop growth have, in fact, been made only to a limited extent, whilst where they occur, precipitates render the corresponding formulae invalid as a guide to actual composition: this latter applies to many experiments in which comparisons have been made between different formulae.[3] Furthermore, the observation that a crop grows well in a particular nutrient solution does not prove that there is not a more favourable composition for that crop.

Since the plant itself excercises certain selective properties, the question may be raised as to the extent to which the composition of the nutrient solution is important for growth and development. However, before pursuing this point further, it is necessary to examine more closely what is meant by "chemical composition". This term comprises (1) the concentrations of the component ions, (2) the total ionic concentration, and (3) pH. Experiments with flowing cultures, in which fresh nutrient solution is supplied continuously to plants, have shown that individual ions are taken up even at very low concentrations, but it is probable that for every ion, there is a certain minimum concentration below which uptake is no longer possible at the required rate. At the other extreme of concentration, luxury consumption may lead to internal toxicity. The point at issue here is whether there is an optimum

concentration of certain ions in solution for a certain crop grown under given environmental conditions, or whether their relative proportions and not absolute concentrations are the determining factors?

Let us assume that absolute concentrations are decisive and that in a particular experiment, optimum concentrations have been found corresponding to *a* me K^+, *b* me Ca^{++} and *c* me Mg^{++} per litre. This gives the ratio of K : Ca : Mg as a : b : c. However, this ratio may also be expressed as a/n b/n c/n, provided the value of a + b + c is given; thus, the optimum composition can be expressed in terms of both a sum and a ratio. The point is that even if the absolute concentrations are considered to be determining factors, the relative proportions of the different ions can also be considered in the same way, if the total ionic concentration is included.

From the author's own investigations along these lines on carnations, tomatoes, and gerberas, it appeared that the relative proportions of the anions were important only within rather wide limits but that the relative proportions of the cations were very important. The total ion concentration is important within the limits $\pm$ 0.2 atmospheres osmotic pressure. In many cases, the pH of the nutrient solution was important within the limits $\pm$ 0.2 pH units.

In the author's opinion, it is essential that in physiological research involving water cultures, the influence of the composition of the nutrient solution be investigated systematically. In order to do this, every possible combination of the variables, anion ratio, cation ratio, total ionic concentration, and pH must be considered.

Superficially, the preparation of nutrient solutions satisfying certain requirements would appear to be straightforward. This however is by no means the case; the pH of the solution, a very important growth factor, introduces serious complications. For example, let us assume that a solution of a certain desired composition has a pH of 5, but that we require a pH of 6.5. This can be achieved by adding an alkali such as KOH. However, a great deal of KOH may be required, especially if the solution contains a large amount of phosphate. At pH 5, practically all the phosphate is present as $H_2PO_4^-$, as shown by the phosophate dissociation curve [1] in Figure 1: in order to obtain a pH of 6.5, 22 per cent of this

$H_2PO_4^-$ must be converted into HPO_4^{--}, according to the dissociation sequence, $H_3PO_4 \rightleftharpoons H_2PO_4^- \rightleftharpoons HPO_4^{--} \rightleftharpoons PO_4^{---}$. The large amount of KOH required for this would therefore, seriously upset the relative proportions of the cations. The answer would be to

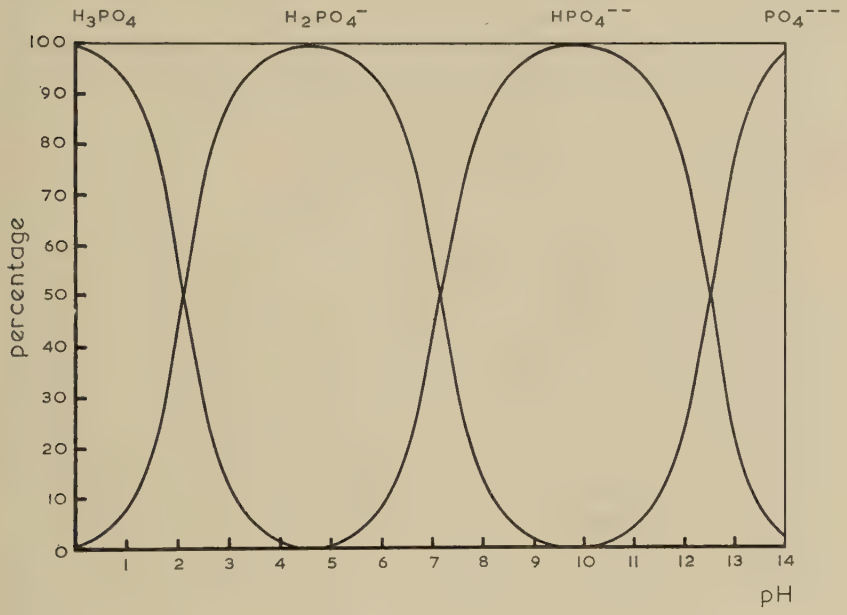

Fig. 1. Phosphate dissociation curve.

adjust the pH by titrating with alkali containing all cations in the same relative proportions as in the original solution; in this case however, the appropriate mixture of KOH + $Mg(OH)_2$ + $Ca(OH)_2$ will only yield a sufficiently dissociated solution of Ca^{++} and Mg^{++} at extremely low concentration.

As an alternative, the desired solution could be prepared with a pH of over 7 by supplying the phosphate as PO_4^{---} and then titrating to the required pH with a mixture of all anions in the same relative proportions as before. The difficulty with this procedure, as will appear later, is that at pH 7, only a limited number of ion combinations are possible without producing a precipitate (*e.g.* of $CaHPO_4$) at an osmotic pressure of 0.7 atm. Since the optimum for many crops must surely not be below 0.7 atm, this method can only be used to a limited extent. Titration with a solution already

containing phosphate precipitates will obviously yield very unreliable results because of the low *dissolution* rates of these phosphates.

The aim of this paper is to determine how a particular nutrient solution can be prepared which satisfies given requirements as to (1) relative cation ratios, (2) relative anion ratios, (3) total ionic concentration, and (4) pH. However, before this problem is considered further, it was thought desirable to investigate how these requirements are met in manurial trials on soils and to what extent, the position can be improved in these cases.

COMPARATIVE MANURIAL TRIALS ON SOILS

If one wishes to investigate, for example, the effect of an addition of potassium to the soil on the development of a particular crop, it is clear that this cannot be realised without introducing other factors, *e.g.* chloride or sulphate. Potassium, at least, must be added as a salt. Although one is inclined to attribute a resulting response to the cation in question, in this case potassium, it may be that the chloride or the sulphate is also involved, besides the increase in the total ionic concentration. Because of the high buffering capacity of the soil, changes in pH do not usually cause trouble.

It is however, possible to avoid these difficulties. One can investigate the influence of potassium with respect to another cation, *i.e.* by working with relative quantities; as already suggested in the introduction to this paper, this is justified even though only "absolute quantities" are evaluated. Thus, in one case, K_2SO_4 may be used and in the other, an equivalent amount of Na_2SO_4; variation can be achieved by supplying the same total quantity of sulphate but distributed to different extents between K_2SO_4 and Na_2SO_4. In this way both the effects of different levels of sulphate and of different total ionic concentrations can be eliminated .The latter, of course, is only possible completely, if salts containing cations of the same valency are used; if in one case, 2 gramequivalents of SO_4'' are supplied as $MgSO_4$, and in the other as K_2SO_4, the former yields 2 gramions and the latter 3 gramions, consequently resulting in a difference in total ionic concentration, though to a smaller extent than with conventional methods.

Curiously enough, the importance of the above does not appear to have been generally appreciated to any real extent. Take for example, experiments involving K : N ratios. If these are made using different amounts of K_2SO_4 and $Ca(NO_3)_2$, their interpretation is not at all straightforward; although only the K : N ratio is usually considered, there are actually four factors to be taken into account – $K^+ : NO_3^-$, $K^+ : Ca^{++}$, $Ca^{++} : SO_4^{--}$ and $NO_3^- : SO_4^{--}$. This complication may be eliminated by applying nitrogen as the ammonium ion. Thus, for the purposes of comparison, the same total amount of $K_2SO_4 + (NH_4)_2SO_4$ is applied, with the ratio

$(NH_4)_2 SO_4$: K_2SO_4 different in every treatment; in this case it is clear that provided that differences in nitrification do not interfere with the results, a valid test is being made of the NH_4^+ : K^+ ratio. If NH_4NO_3 is used for increasing the N : K ratio however, the total ionic concentration is affected; it must also be pointed out that on certain soils, the use of ammonium salts in such experiments may lead to considerable changes in pH.

Many more difficulties are encountered in manurial experiments on soils, but the few examples given above serve the purpose of illustration.

THE PROBLEM WITH NUTRIENT SOLUTIONS

Let us now take the case of a nutrient solution corresponding to a particular formulation, in which the potassium content is to be varied. This may be done by the addition of extra potassium but it is clearly impossible to do this without affecting the pH. In order to maintain the pH at a desired value, the addition of an anion is essential, *e.g.* sulphate or chloride. As a result of this, of course, the effect of the sulphate or chloride must be considered besides that of the potassium. In the case of nitrogen, attempts can be made to avoid this effect by adding NH_4NO_3, but many plants react differently to nitrogen when supplied as nitrate or ammonium ion; also, because of the more rapid uptake of ammonium by the plant, there may follow an appreciable drop in pH, in which case, if the pH is to be maintained, a cation must be added which may interfere with the experiment.

Systematic research into the influence of the chemical composition of the nutrient solution is only justified if one deals with relative ratios of the nutrients. There is no objection whatsoever to this approach. As already discussed in the introduction, whatever the values assigned to the "absolute" levels, it is still possible to use relative ratios, provided that the total ionic concentration and also the pH, are taken into account. This does not imply, as was suggested in the previous discussion on soil, that K_2SO_4 can be replaced by Na_2SO_4; in both cases, the total ionic concentration is increased.

Consideration of the ratio, N (as NO_3) : P : S : K : Ca : Mg is meaningless since the ratio [N(as NO_3) + P + S] : [K + Ca + Mg] is already determined by the pH. Hence one must deal with different relative ratios of anions in combination with different relative ratios of cations. Many people attach great importance to the K/N ratio; although the significance of this is open to argument [5], it can also be considered on the basis of relative ratios, since high K^+ relative to the other cations and low NO_3^- relative to the other anions means the same as a high K/N ratio. In principle, using a

systematic approach, it is desirable to take into account as many cations and anions as possible. In this article attention has been rectricted to the anions, NO_3^-, $H_2PO_4^-$ (HPO_4^{--} and PO_4^{---}) and SO_4^{--} and to the cations, K^+, Ca^{++} and Mg^{++}.

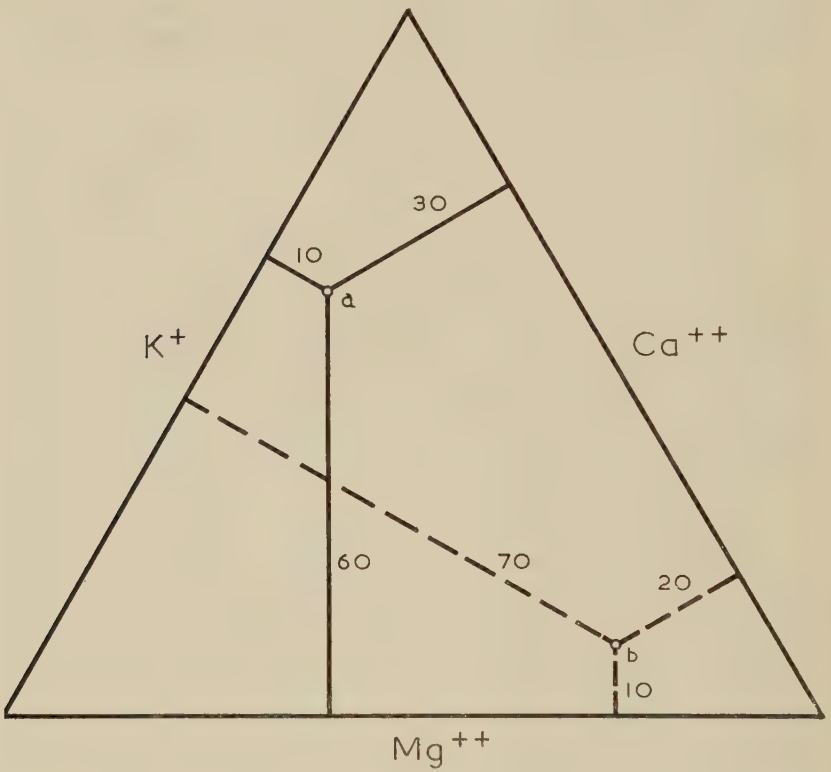

Fig. 2. K^+ : Ca^{++} : Mg^{++} ratios represented by the points a en b.

The representation of relative ratios as single points within a triangular system offers considerable advantages. This can be done by making the ratios correspond to the length of the perpendiculars from each point to the 3 sides of an equilateral triangle, each side representing the proportions of a particular ion (*cf* Fig. 2); the ratio of the 3 ions is then easily determined by reference to the relative positions of the points within the triangle. For convenience, each side is divided into 10 equal parts and a triangular co-ordinate system imposed. Referring to Figure 3, every point on the side AB for example corresponds to 0 per cent Mg^{++}, those on the line

CD to 20 per cent Mg^{++}, and so on; point *N* corresponds to a ratio of 20 per cent K^{+}, 30 per cent Ca^{++}, and 50 per cent Mg^{++}. Naturally one can consider each ratio (or point) as a ratio of weights, as an ion ratio, or as an equivalent ratio. Because of the connection with

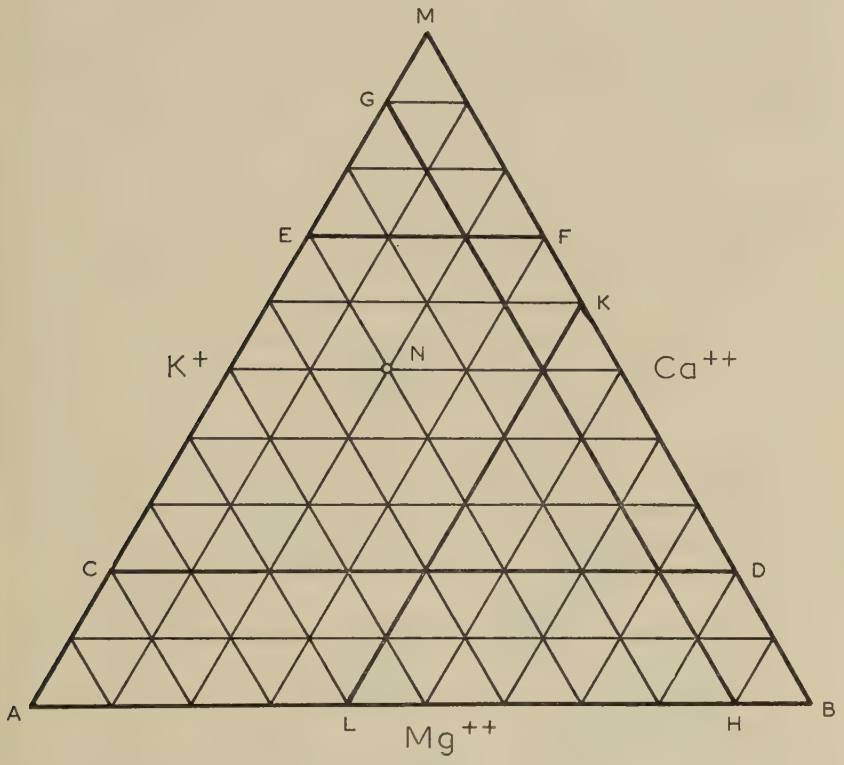

Fig. 3. Triangular graph used for comparing the K^{+} : Ca^{++} : Mg^{++} ratios.

pH adjustments to be discussed later, we prefer equivalent ratios. By locating all points within a cation and an anion triangle and combining one from each, it is possible to classify systematically, all possible nutrient combinations.

For nutritonal-physiological investigations each combination must correspond to a particular "formula" according to which a certain ionic concentration and a certain pH are obtainable without affecting the particular ratio used. Satisfying the pH requirement represents a serious stumbling block. For example, let us assume that nutrient solutions are to be prepared according to two formu-

lae giving a particular total ionic concentration. If all the phosphate is supplied as $H_2PO_4^-$, the solutions will have low pH values (about 5). Higher pH values may be obtained by titration with KOH, but then the K : Ca : Mg ratio must be readjusted *i.e.* the amount of KOH required must be converted into the same K : Ca : Mg ratio as in the original solution; at the same time, the total ionic concentration, increased by the titration, has also to be restored. The result is that the pH is no longer the same as immediately after the titration.

The problem of this pH change calls for a theoretical treatment of incomplete dissociation and pH with regard to $CaHPO_4$, $MgHPO_4$, $Ca(OH)_2$ and other compounds. This is beyond the scope of the present article and will not be discussed here. The result of such a treatment however, indicates that there must be a systematic relation between the amount of OH ions required to give the desired pH, the absolute concentration of phosphate and certain cation ratios in the solution.

It will be seen later that by means of certain curves, it is possible to fit into a formula, any chosen combination and that in this way, the following requirements can be satisfied with considerable accuracy: –

(1) a particular relative cation ratio
(2) a particular relative anion ratio
(3) a particular total ionic concentration
and (4) a particular pH.

Since the actual course of these curves in such a system has to be determined experimentally, a large number of solutions must be prepared.

REQUIRED COMBINATIONS OF ION RATIOS, TOTAL IONIC CONCENTRATION AND pH

Even supposing the approach described above was not entirely successful, the formulae obtained may yet find application to physiological studies on nutrition. For example, it would be possible to make a selection from those formulae giving a suitable pH, even if this did not exactly correspond to the one required. It was therefore considered desirable to include in this investigation, a large number of 'combinations'; these would also provide an even better basis for the construction of the above-mentioned curves.

Following this principle, we have tried to include as many ratios as possible; assuming 50 for the anions and 50 for the cations, this gives us 50 × 50 = 2,500 possible combinations. If, in addition to these, we wish to include say, 5 different total ionic concentrations and 5 different pH values, the total number of solutions required would be 62,500. Although these will include many combinations that can be rejected on theoretical grounds because of expected precipitates, there still remains too large a number to be dealt with in practice. The number of ratio combinations must therefore be reduced or certain ranges of ratios within the triangle omitted. The phosphate content of nutrient solutions used in practice, expressed as milliequivalents * $H_2PO_4^-$, generally does not exceed 25 per cent of the total content of anions.

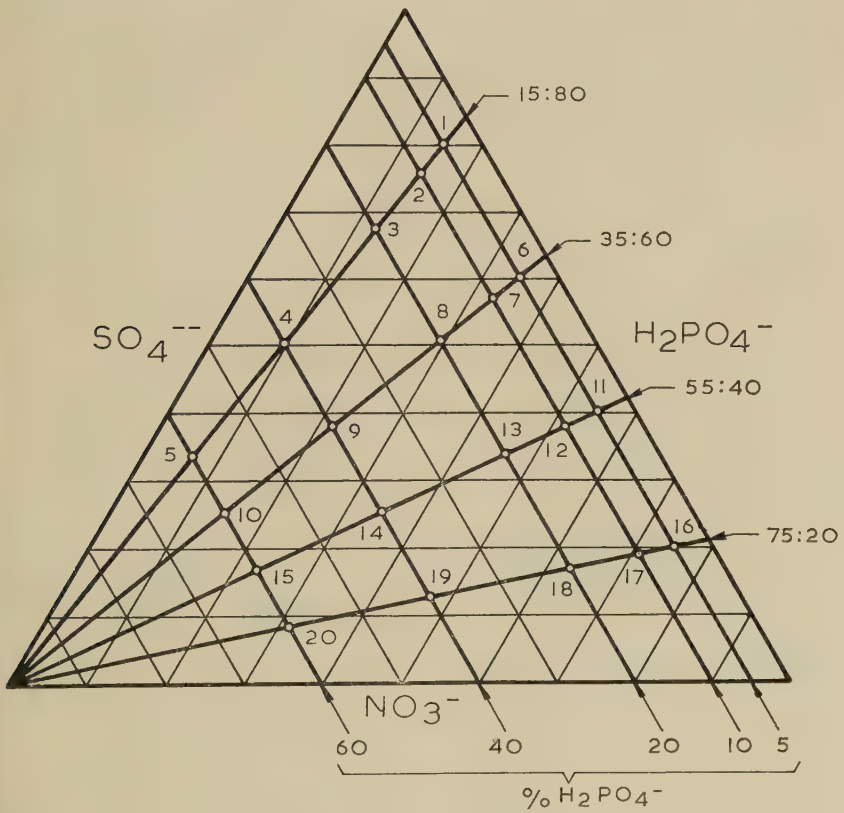

Fig. 4. The anion ratios used in the investigation. Combinations 1 to 20.

* In this article, phosphate is always expressed as milliequivalents-percent, or as milliequivalents (me) of H_2PO_4 even though, depending on the pH, it may be partly present as HPO_4. Thus, although the addition of 2 me H_2PO_4 to a solution may for example, yield 1 me H_2PO_4' and 2 m.e. HPO_4'', this still amounts to 2 mg ions of phosphate. Confusion will be avoided if the phosphate is always expressed as me of H_2PO_4.

Although it is not desirable to exclude entirely the higher proportions of phosphate because these may be of fundamental physiological interest, attention can be largely directed to the lower proportions. In any case, high proportions of phosphate will allow for only a few combinations because of precipitation *e.g.* of $CaHPQ_4$.

To begin with we have chosen phosphate contents corresponding to the 5, 10, 20, 40, and 60 per cent lines in Figure 4. Combined with four different NO_3^- : SO_4^{--} ratios, *viz* 80 : 15, 60 : 35, 40 : 55 and 20 : 75, these give 20 different anion combinations (Nos. 1 to 20, Fig. 4). The 16 cation ratios chosen for investigation are shown in Figure 5 (a–p); since it is already known that best results are obtained with low proportions of Mg, attention has been mainly focussed on these.

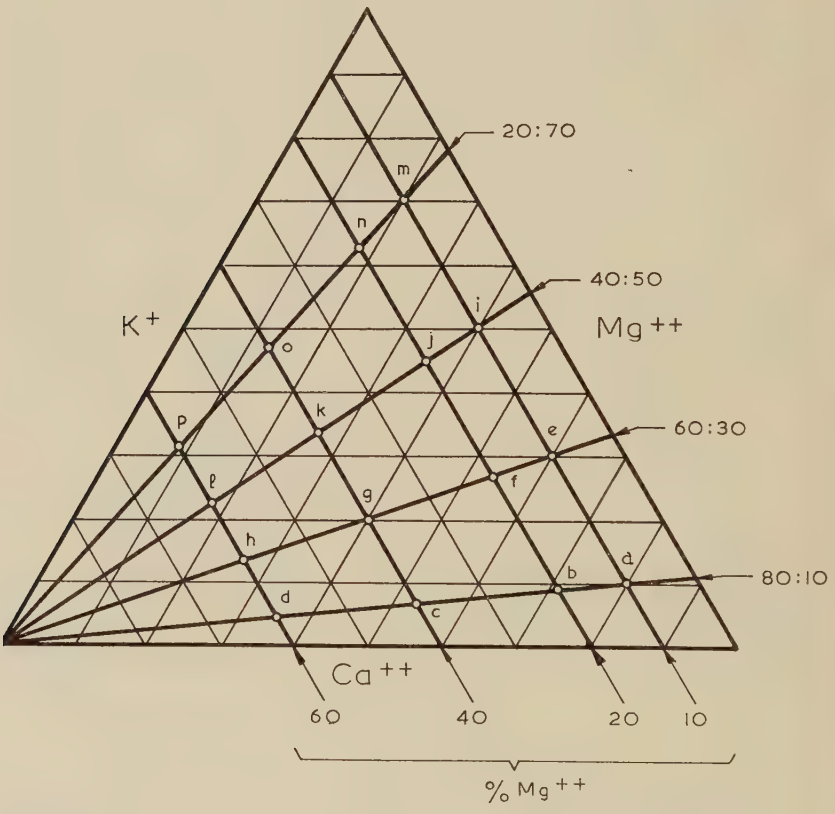

Fig. 5. The cation ratios used in the investigation. Combinations a to p.

The 20 anion ratios and 16 cation ratios together yield 320 combinations. With 5 different pH-values included, namely 5.0, 5.8, 6.5, 7.1, and 7.6, the total amounts to 1600 different combinations.

Obviously, if the effects of different ionic ratios are to be distinguished,

the total 'strengths' of the solutions must be comparable. This raises the question as what "strength" actually means to the plant. Clearly it is not the weight of the salts but their ionic equivalents; to some people however this is a disputable matter. However, the calculations necessary to ensure comparable total equivalent contents become very complicated, especially when allowance has to be made for adjustments to the pH value by titration; among other factors, this is bound up with the phosphate dissociation series, $H_3PO_4 \rightleftharpoons H_2PO_4^- \rightleftharpoons HPO_4^{--} \rightleftharpoons PO_4^{---}$. Partly because of this complication, but largely due to the fact that earlier experiments with different crops have demonstrated the great importance of total ionic concentration, as reflected in the osmotic values of the nutrient solutions, the total ionic concentration has been kept constant. Because of the already very large number of investigations entailed, we have therefore restricted ourselves to a single concentration, one in which very many plants are known to thrive, corresponding to 0.71 atm osmotic pressure. Assuming a theoretically ideal complete dissociation at 15°C, this is equivalent to a total concentration of 30 mg ions per litre, provided no hydrolysis or precipitation occurs.

The 1600 combinations have been incorporated into formulae and subjected to laboratory investigation. A detailed description of their preparation is given below.

PREPARATION OF THE TEST SOLUTION COMBINATIONS

In order to clarify the procedure employed, full details are given in Table I for the Solution *1a*, corresponding to the Anion Ratio No. *1* (*cf* Fig. 4) and Cation Ratio *a* (*cf* Fig. 5), with an osmotic pressure of 0.71 atm.

In the Column (*1*) the equivalent content of each ion is expressed as a percentage of the corresponding total anion or cation

TABLE 1

Combination	Ratio		Absolute values	
1a	equiv. in % (*1*)	ions (*2*)	mɛ per litre (*3*)	mg ions per litre (check) (*4*)
NO_3^-	80	80	13.151	13.151
$H_2PO_4^-$	5	5	0.822	0.822
SO_4^{--}	15	7.5	2.466	1.233
Total	100		16.439	
K^+	80	80	13.151	13.151
Ca^{++}	10	5	1.644	0.822
Mg^{++}	10	5	1.644	0.822
Total	100	182.5	16.439	30.001

equivalent content, just as in Figures 4 and 5. In the Column (*2*) these values have been converted to mg ions; this column totals 182.5. For an osmotic pressure of 0.71 atm, the total ionic concentration must be 30 mg ion per litre. The absolute amounts of each ions as milliequivalents per litre are obtained by multiplying the values in the first column by 30/182.5 (giving a total of 30 mg ions per litre); the values are given in Column (*3*). As a check, these values have been re-converted in Column (*4*) into mg ions per litre, and the required total of 30 is confirmed.

Summing up, it will be seen that Column (*3*) gives the number of milliequivalents per litre of each ion corresponding to a particular relative ratio of anions and of cations and a total ionic concentration of 30 mg ions per litre. Thus, except for pH, the solution prepared according to this formula satisfies all requirements.

In order to apply the formula to actual salts, a scheme has been devised which enables the procedure to be followed systematically and which utilizes the smallest number of different salts, giving preference to those which are easily soluble. For most formulae this has meant 5 different salts, but there is a smaller group requiring 4 salts and a very small group, 6 salts. Details of the system applied to solution Combination *1a* are outlined in Table 2.

TABLE 2

Combination *1a* (30 mg ions/l)			Milliequivalents per litre					
			K^+	Ca^{++}	Mg^{++}	NO_3^-	$H_2PO_4^-$	SO_4^{--}
Salt	Normality (*N*)	ml per 10 l	13.151	1.644	1.644	13.151	0.822	2.466
KH_2PO_4	1	8.22	0.822				0.822	
$Ca(H_2PO_4)_2.1aq.$	0.1							
$Mg(H_2PO_4)_2.3aq.$								
$Ca(NO_3)_2.4aq.$	10	1.644		1.644		1.644		
$CaSO_4$	0.02							
$MgSO_4.7aq.$	2	8.22			1.644			1.644
$Mg(NO_3)_2.6aq.$	1							
KNO_3	1	115.07	11.507			11.507		
K_2SO_4	1	8.22	0.822					0.822

As far as possible, the required number of milli-equivalents of the various ions are obtained preferentially from salts in the order, top to bottom; this order is based first on the ease with which the phosphate can be supplied in manageable form, *viz* $K > Ca > Mg$. The balance of calcium is then made up in the order $NO_3^- > SO_4^{--}$,

and that of magnesium in the order $SO_4^{--} > NO_3^-$; any nitrate outstanding is then made up from KNO_3. Final amendments, if necessary, are automatically made up by adding equivalent amounts of K^+ and SO_4^{--}.

To facilitate the preparation of these solutions, calculations have been made to determine how many ml of stock salt solution of given normality are needed per 10 litres of nutrient solution.

Attention is drawn to the difficulty of obtaining a clear solution of 0.02*N* $CaSO_4$ if $CaSO_4$ is used: this can be avoided by dissolving 1 g $CaCO_3$ in 200 ml 0.1*N* H_2SO_4 and making up to 1 l with water *after* it has dissolved.

In each case, 10 l of the required nutrient solution were prepared using demineralized water (spec. conductivity usually 10^{-6} and never $< 10^{-5}$ $ohm^{-1}cm^{-1}$). The solution was then thoroughly aerated for 24 hours so as to obtain equilibrium with air. The pH of the resulting solution was measured with a Radiometer pH meter, Type M22P: all preparations were carried out in the laboratory at a temperature of usually, about 21°C.

Solution *1a* had a pH value of 4.76 which, after 24 hours aeration, rose to 5.33. One litre of this solution was then titrated to pH 5 with HNO_3; the titrations were made with continuous measurements of pH, and agitation by magnetic stirrer. 0.14 ml. of HNO_3 (.0838*N*) were found necessary to obtain a pH of 5; this is equivalent to 0.012 me NO_3^- per litre. Further aliquots of 1 l nutrient solution were titrated to obtain the other pH values – 5.8, 6.5, 7.1, and 7.6; the amounts of KOH required corresponded respectively to 0.060, 0.296, 0.606 and 0.772 me K^+. After each titration, the solutions were again aspirated for 1 hour and the pH remeasured; these gave new pH values of 5.03, 5.86, 6.45, 6.85 and 7.30 respectively. Since it was decided to accept only a deviation of $\pm$ 0.1 pH units from the required pH value the solution giving a pH of 6.85 instead of 7.1 $\pm$ 0.1 was again titrated with KOH to pH 7.1; this required a further 0.65 ml of KOH (.0837 *N*) = 0.054 me K^+. After aspirating again for 1 hour, the pH fell to 7.0. The solution giving a pH of 7.30 instead of 7.6 $\pm$ 0.1 was similarly retitrated with KOH and an equivalent of 0.087 me K^+ was required in this case; after 1-hour's aspiration, the pH rose to 7.34, indicating incomplete dissociation. Further titrations were made and finally, after a total of 0.949 me K^+ had been added, a solution was obtained which

after aeration, gave a pH of 7.54 *i.e.* within the range 7.6 ± 0.1.

Although the solutions at the two highest pH values were completely clear visually, there was some indication, as mentioned above, that dissociation was incomplete. Note has been taken of this together with the observation that some of the solutions were opalescent or actually turbid. However, all 320 combinations have been treated in the same way even where incomplete dissociation or precipitation had been expected on theoretical grounds. Although the actual theoretical calculations are beyond the scope of this paper, it can be said that they have given good agreement with the results.

The procedure described so far gives solutions satisfying the pH requirements but, of course, alters the relative proportions of the anions (where HNO_3 was added) or of the cations (where KOH was added). Furthermore, the total ionic concentration now exceeds the stated 30 mg ions per litre. In order to rectify the ratios the amounts of NO_3^- or K^+ added to adjust the pH have been converted to equivalents of NO_3^-, $H_2PO_4^-$ and SO_4^{--} in the one case or K^+, Ca^{++} and Mg^{++} in the other, in the same proportions as in the original solution. The method of conversion is illustrated below for Solution *1a* (Table 1) with a pH of 6.5 (actually 6.45); to obtain this pH, 0.296 me K^+ (similarly OH') had to be added per litre of solution. The conversion factor is obtained from the ratio of the total me cations (including the extra K^+) to the original total me cations: –

$$\text{Conversion factor} = \frac{13.151 + 1.644 + 1.644 + 0.296}{13.151 + 1.644 + 1.644} = 1.018005$$

1.018005×13.151 me $K^+ = 13.388$ me K^+
1.018005×1.644 me $Ca^{++} = 1.674$ me Ca^{++}
1.018005×1.644 me $Mg^{++} = 1.674$ me Mg^{++}

These converted values now give the same relative proportions of K, Ca, and Mg as in the original solution and the new formula becomes:

In	K^+	Ca^{++}	Mg^{++}	NO_3^-	$H_2PO_4^-$	SO_4^{--}	Total
me	13.388	1.674	1.674	13.151	0.822	2.466	–
mg ions	13.388	0.837	0.837	13.151	0.822	1.233	30.268

However, the total ionic concentration has been increased to

30.268 mg. ions per litre and the formula must therefore be readjusted to give 30 mg. ions. The final result is: –

In	K^+	Ca^{++}	Mg^{++}	NO_3^-	$H_2PO_4^-$	SO_4^{--}	Total
me	13.269	1.659	1.659	13.035	0.815	2.444	–
mg ions	13.269	0.830	0.830	13.035	0.815	1.222	30.001

The amounts of the various ions can now be derived from the appropriate salts according to the procedure outlined earlier, making sure that the required surplus of cation (K) is in the first place, made up entirely from KOH; although the surplus cation could also have been supplied as $Ca(OH)_2$ or $Mg(OH)_2$, KOH is preferred because of its high solubility. The actual surplus of cations in the final solution amounts to $(13.269 + 1.659 + 1.659) - (13.035 + 0.815 + 2.444) = 0.293$ me; this is added as KOH after which the remaining equivalent amounts of cations and anions are added as described earlier.

Ten litres of Solution *1a* with pH 6.5 were now prepared according to this new formula. After aeration the pH changed to 6.58; it is clear that this change in pH has taken place because of the change from K^+ to $K^+ + Ca^{++} + Mg^{++}$. This example, in which 0.815 me $H_2PO_4^-$ were present and a surplus of 0.293 me OH^- had to be added, will be further discussed later on.

Except for those solutions already turbid or giving a precipitate on titration with KOH, all 320 ion combinations have been converted in this way and tested according to the above scheme at 5 different pH values.

COMPUTATION OF FORMULAE

As mentioned earlier there is apparently a systematic relation between the amount of $H_2PO_4^-$ present in the solution and the surplus OH ions which must be added in order to obtain a certain pH. This relationship however, is influenced by the relative cation ratios, especially those between K^+ and Ca^{++}. The pH depends largely on the ratio of OH^- to $H_2PO_4^-$ ions and this may be depicted by a curve which is displaced according to the K : Ca ratio.

In the 360 ion combinations, only four K : Ca ratios were involved, namely, 80 : 10, 60 : 30, 40 : 50 and 20 : 70; however, tests were made on another 80 combinations giving intermediate

K : Ca ratios so as to determine with some accuracy, the influence of this ratio on the displacement of the $OH^-/H_2PO_4^-$ curves.

In Figure 6, the pH (abcissa) is plotted against the amount of

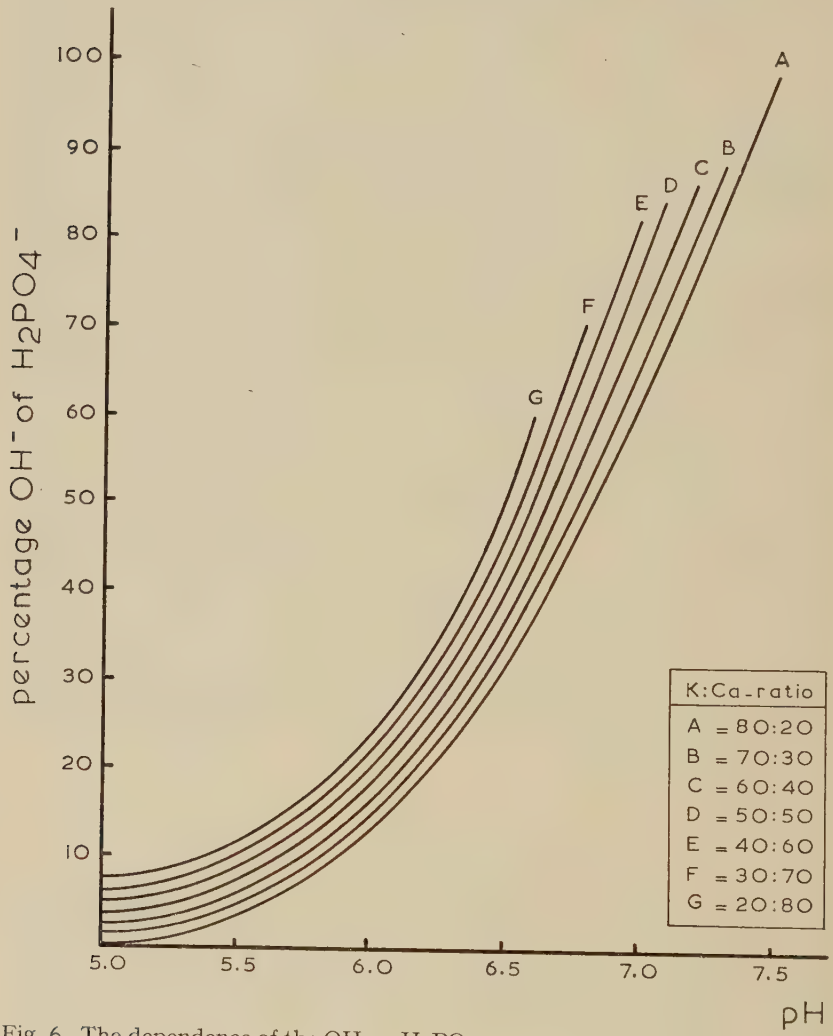

Fig. 6. The dependence of the OH^- : $H_2PO_4^-$ curves upon the K : Ca ratio.

OH^- expressed as a percentage of the amount of $H_2PO_4^-$ in the solution (ordinate); curves are given for 7 different K : Ca ratios (A–G), and intermediate values can be obtained by interpolation. The curves illustrated however are on too small a scale for practical

purposes and larger scales are available for those who wish to use them.

With the aid of these curves, it is now possible to work out any desired formula. Suppose we require a ratio (in equivalents) of NO_3^- : $H_2PO_4^-$: SO_4^{--} = 5 : 3 : 1 (*i.e.* 55.556 : 33.333 : 11.111 to give a total of 100) and a ratio of K^+ : Ca^{++} : Mg^{++} = 50 : 30:20, with a total ionic concentration of 30 mg ions and a pH of 6.3.

The ratio K : Ca = 5 : 3 or 62.5 : 37.5 (to give a total of 100) and the line C in Figure 6, representing a ratio of 60 : 40, is the nearest to this. For pH 6.3 this would indicate an OH' requirement of 26.9 per cent of the phosphate content. Interpolation between curves C and B to correspond with the actual 62.5 : 37.5 ratio, indicates a somewhat lower value, namely, 26.0 per cent; this is the value adopted.

In the desired solution, $H_2PO_4^-$ amounts to 33.333 per cent of the total equivalent anion content so that the OH^- corresponding to 26.0 per cent of this, amounts to 8.667 OH ions. These OH ions are now divided among the cations in the same proportion as the cation ratio, *i.e.* 50 : 30 : 20. This yields 4.333 K^+, 2.600 Ca^{++} and 1.733 Mg^{++} and when these amounts are added to the corresponding components of the original cation ratio (total 100), the ratio still holds good. Thus, both cation and anion contents have now been expressed in terms of milliequivalents: –

K^+	Ca^{++}	Mg^{++}	NO_3^-	$H_2PO_4^-$	SO_4^{--}
54.333	32.600	21.733	55.556	33.333	11.111

In order to obtain a total ionic concentration of 30 mg ions, the above values are first converted into mg ions: -

K^+	Ca^{++}	Mg^{++}	NO_3^-	$H_2PO_4^-$	SO_4^{--}	Total
54.333	16.300	10.867	55.556	33.333	5.556	175.945

Since the total of 175.945 has to correspond to 30 mg ions, the final formula is obtained by multiplying throughout by 30/175.945. The result is a formula for a nutrient solution satisfying the following conditions: –

(1) a desired relative anion ratio
(2) a desired relative cation ratio
(3) a desired total ionic concentration

and (4) a desired pH with a maximum tolerance of $\pm$ 0.1 pH.

Assuming that the nutrient solution is prepared according to this formula, the pH after aeration will be about 6.37 *i.e.* only 0.07 units higher than required.

In the case of solution 1*a*, which has been given earlier as an example, the amount of OH^- added (0.293 me) corresponds to 35.9 per cent of the amount of $H_2PO_4^-$(0.815 me) present in the solution; according to curve A in Figures 6, this would indicate a pH of about 6.58, which agrees exactly with that actually found.

Every possibly ion combination having a total concentration of 30 mg ions, which does not produce a precipitate, can be prepared in this way within a pH range covered by the K : Ca ratios Precipitates, if any, and the significance of the above-mentioned pH range will be discussed later.

Regarding the extent to which the pH of the solution satisfies the required value, it must be stated that no better accuracy than $\pm$ 0.2 pH units can be guaranteed within the range 5.0 to 5.5. This is due to the fact that between these values, the phosphate dissociation curve runs practically horizontal, in other words, the smallest change in OH^- concentration produces a large change in pH. Between pH 5.5 and 6.0, the precision is $\pm$ 0.15 and between pH 6.0 and 7.0, $\pm$ 0.1. Because of the incomplete dissociation of $CaHPO_4$, and other salts above pH 7,0, the accuracy falls to $\pm$ 0.2.

The method does not hold for extreme values or for solutions not containing calcium or phosphate.

It will be observed in Figure 6 that the curves cover only a certain pH range with a minimum at pH 5.0; relationships below this value were, in fact, not investigated. However, between pH 4.0 and 5.0, there is little point to the investigation since the dissociation curve for $H_2PO_4^- \rightleftharpoons HPO_4^{--}$ then runs horizontally and in this region the solution is no longer buffered by phosphate. Below pH 4.0, the accuracy increases again (*cf* Figure 1). Above the pH range covered by the curves, few solutions are possible with a total ionic concentration of 30 mg ions per litre; only in the case of extreme ratios are higher pH values possible *e.g.* in solutions containing very little Ca. This is because of the occurrence of turbidity or actual precipitation. Nevertheless, a few solutions are still possible with pH values greater than those indicated by the curves, but these require more OH ions than would normally be necessary to achieve the desired pH. In such cases, the dissociation

of certain ion combinations is also very incomplete and a stage is finally reached when the addition of OH ions does not produce any rise in pH but even a fall, because of precipitation of $CaHPO_4$ and other compounds. Even within the pH range covered by the curves, certain combinations are also not possible because of precipitation. On the whole, about 58 per cent of all possible combinations can be used around the point where the appropriate curve ends. Also near the 'lower' regions of the curves, a number of combinations are not possible; for Curve A, this involves about 15 per cent of all possible combinations at pH 7.1 and for Curve C, about 15 per cent at pH 6.5.

Whether a particular combination at a certain pH is possible or not, can be determined with some precision. No combination with the product of Ca^{++} and SO_4^{--} greater than 60 mg ions per litre is possible, independent of the pH of the solution. However, this concerns only a few combinations; in Figures 4 and 5, it applies only to the Anion Ratios *16* and *17* in combination with Cation Ratios *m* and *n*. Solutions with the product of Ca^{++} and HPO_4^{--} amounting to about 2.2 mg ions per litre are also doubtful; if $Ca^{++} \times HPO_4^{--}$ is greater than 2.2, the solution becomes turbid. There are however, border-line cases in which precipitation may or may not occur depending on the other ratios. The absolute contents of Ca^{++} and total phosphate are readily determined from the formula calculated; the absolute content of HPO_4^{--} may then be found from its percentage of the total phosphate read from the phosphate dissociation curves in Figure 1, at the appropriate pH.

The method described above holds good for a total ionic concentration of 30 mg ions per litre. Although the immediate objective of this investigation has therefore been achieved, tests have also been made on solutions with a total ionic concentration of 20 mg. ions per litre (0.47 atm osmotic pressure). In these cases also, the curves can be used with a certain accuracy. No tests have been made on solutions with lower concentrations; in any case, this must be looked upon as a separate investigation since the curves need then to be extended to regions of higher pH. At these lower concentrations, many more combinations are possible, especially at the higher pH values.

When the total ionic concentration is raised above 30 mg ions per litre, only a limited number of combinations are possible. Thus,

at 1 atm osmotic pressure (about 42 mg ions per litre), only a few nutrient solutions can be made, *viz.* only the cation combinations *a*, *b*, *c* and *d* (Figure 5) in combination with certain anion ratios. Above pH 6.5, only very few extreme ion ratios are still possible.

In this publication, only 3 anions and 3 cations have been investigated. Tests have been made as to the possibility of including a fourth cation, Na; in this case, the same K : Ca ratio can be used, but taking (K + Na) : Ca into account instead, i.e. the sum of sodium and potassium is treated as potassium. As far as we have been able to determine, this does not upset the reliability of the method. The calculations involving four cations are identical with those for three. No investigation has yet been made as to whether NH_4 ions, for example, can be dealt with by the same curves; in this case much more attention would be necessary to those combinations producing precipitates.

SUMMARY

So as to provide a basis for nutritional and general physiological experiments on plants, a systematic investigation has been made into the preparation of nutrient solutions satisfying certain requirements as to the relative ratios of the nutrient ions, the total ionic concentration and the pH, without complications due to precipitation. A method has been developed whereby it is possible to compute in advance, a formula for the composition of the nutrient solution which satisfies these requirements.

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THE TOXICITY OF LEGUME SEED DIFFUSATES TOWARD RHIZOBIA AND OTHER BACTERIA

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INTRODUCTION

The practice of inoculation of seeds with legume bacteria or the more controversial "azotobacterin", "phosphobacterin" and other bacterial fertilizers (Cooper [4]) calls for an understanding of survival and growth of micro-organisms on seeds. While something is known of stimulation of organisms around seeds (Picci [15]) very little is known of bacterial inhibition by germinating seeds.

In the last twenty years considerable attention has been paid to the presence of antimicrobial substances in plants so that active substances have now been found from at least 157 families (Nickell [13]). In some instances such substances have been extracted from seeds, *viz.* Ivanoics and Horvath [11] found an antibacterial substance, raphanin, in radish seed and Osborn and Harper [14] reported the exudation from germinating *Leptosyne maritima* seed of a toxin toward *Staphlococcus aureus*. Most of the study of antibiotics in seeds and other plant parts is of only indirect interest to the plant scientist since animal pathogens have been used as test organisms by most investigators and antibiosis has been studied mostly with extracts of macerated plant parts.

There are, however, some investigations to which these objections do not apply. Ferenczy [5] in screening 512 plant species from 88 families found that seed coats of 52 species from 19 families contained antibiotics to the gram-positive soil bacteria *Bacillus cereus* var. *mycoides*, *Bac. megatherium* and *Bac. subtilis* but only one produced even slight inhibition toward the gram negative *Aerobacter aerogenes*, *Erwinia carotovora* and *Xanthomonas malvacearum* and a further two against *E. carotovora* and *X. malvacearum* but not *A. aerogenes*. No legumes were included in the tests. Ark and Thompson [2] found that diffusates and extracts from wheat and barley were active against five gram-positive bacteria and twelve plant pathogenic fungi but not against gram-negative bacteria. They regarded seed diffusates as a

defence mechanism against infection. Garber and Houston [8] reported the presence of an inhibitor to *Verticillium albo-atrum* in seed coats of both wilt susceptible and resistant cotton varieties. Antibiotic action against the gram-positive plant pathogenic bacterium *Corynebacterium fascians* was studied in detail by Jacobs and Dadd [12] who found inhibition by diffusates from the seed coats of a range of legumes and other plants. Strains of *C. fascians* varied in their sensitivity. The possible ecological importance of toxic seed diffusates was demonstrated in that an inverse relationship existed between the sensitivity of a strain of *C. fascians* to sweet-pea diffusate and its ability to infect sweet-pea seedlings under laboratory conditions.

Information on the effects of seed diffusates on *Rhizobium* (gram negative) is slight. Vincent [21] presented circumstantial evidence of two workers indicating a seed toxicity to rhizobia, *viz.* Greenwood observed a better survival of inoculum applied in a peat-pumice carrier than in a milk suspension and McLeod found a more rapid death of legume bacteria on clover seed than on glass beads. Earlier workers had noted a rapid decline of rhizobia on seed (Fred, Baldwin and McCoy [6]). Thompson [19] has shown that extracts of subterranean clover seed coats contain a thermostable, water-soluble, antibiotic effective against a strain of *Rhizobium trifolii*. The activity of diffusates from white clover and lucerne was much less.

Knowledge of antibiotic seed diffusates up to the present may be summarized thus: Antibiotic seed diffusates occur with a wide range of plants and these are active against a number of plant pathogenic fungi and gram-positive bacteria. Activity against gram-negative bacteria appears to be rare and seeds of only six plant species have been shown to be active against a total of four gram-negative bacteria. Almost nothing is known of the role of antibiotic seed diffusates in determining the survival in soil of organisms inoculated to seed and in the determination of the composition of the microflora of the germinating seed.

The present investigations arose from an observation of toxicity toward Rhizobium strains by seed diffusate prepared from the tropical pasture and cover-crop legume *Centrosema pubescens*. They include tests of seed diffusates from three legumes against a range of rhizobia and other soil bacteria and studies of the effect of seed diffusates on micro-organisms around seed germinating in sand and soil.

MATERIALS AND METHODS

a. *Seeds*

The seeds used were from *Centrosema pubescens* Benth., subterranean clover (*Trifolium subterraneum* L. Bacchus Marsh strain) and lucerne (*Medicago sativa* L. Hunter River strain).

b. *Seed sterilization*

Seed was shaken for ten to fifteen minutes in a solution of equal parts of ethyl alcohol and 20 volume per cent hydrogen peroxide followed by three washings with sterile, distilled water over a further ten minutes. Tests showed no residual toxicity from the sterilizing agents.

c. *Preparation of seed diffusate*

Five hundred grammes of surface-sterilized seed were soaked in 700 ml of sterile distilled water for 18 hours at 20°C and the diffusate subsequently decanted.

d. *Agar-plate testing for inhibition*

Test organisms were inoculated onto the surface of a mineral salts—yeast extract—mannitol base (Allen [1]) solidified with 0.8% agar (YM agar). With each seed type 10 surface-sterilized seeds were placed on each of three agar plates, incubated at 25°C and observations made of growth inhibition at two to four days depending on the rate of growth of test organisms. The extent of inhibition was assessed by measuring the distance from the seed to the edge of the zones of inhibition of bacterial growth.

e. *Counts of organisms from seeds and glass beads*

Each of ten seeds or glass beads was shaken vigorously in ten ml of sterile tap water and the viable count of a one-ml aliquot made using the pour plate method with YM agar.

RESULTS

Initial observations

Seed diffusate prepared from *C. pubescens* and sterilized by steam at 120°C for 20 minutes failed to support growth of 3 Rhizobium strains from *C. pubescens* (Rhizobium QA 523, QA 549 and QA 616) one strain from each of *Glycine javanica* (Rhizobium QA 619), lucerne (Rhizobium QA 383) and clover (Rhizobium TA 1), and *Agrobacterium radiobacter*. Addition of diffusate to YM broth prevented growth. The same results were obtained when the diffusate was sterilized by steaming at 100°C for an hour on three successive days or by filtration through a Seitz-E–K pad. The pH of seed diffusate was 6.1 and of YM broth with diffusate added, 6.6.

It was concluded that *C. pubescens* seed diffusate included a thermostable, water-soluble, toxin-inhibiting rhizobia and other organisms. The toxicity could not be attributed to acidity. However, the arbitrarily selected concentration of the prepared seed diffusate made relation of the results to behaviour of single seeds difficult.

The occurrence of seed toxicity with different legumes and Rhizobium strains

Surface-sterilized seed of *C. pubescens*, clover and lucerne were tested against 17 Rhizobium strains isolated from a wide range of legumes. Inhibition from lucerne seed was slight, only very narrow inhibition zones (0–0.5 mm) being observed. All clover seeds produced inhibition zones (Plate 1) but inhibition from *C. pubescens* occurred only around swollen and germinating seeds (Plate 2). Non-inhibition by hard seeds of Leguminosae is well known (Toole, Hendricks, Borthwick and Toole [20]). Table 1

TABLE 1

Inhibition of Rhizobium strains by diffusates from *C. pubescens* and subterranean clover

Organism	Host of isolate	Radius of inhibition *, mm			
		C. pubescens		Subterranean clover	
		Complete	Partial	Complete	Partial
Tropical legume miscellany					
QA 869	*C. pubescens* Benth.	1.5	3	2	4
QA 845	,,	1.5	2	0.5	1.5
QA 616	,,	1	2	2	5
QA 549	,,	1.5	3	1	3
QA 618	*Glycine javanica* L.	1.5	3.5	1	3
QA 862	*Phaseolus lathyroides* L.	1	3	2	4
QA 864	*Psoralea eriantha* Benth.	2	5	2	5
QA 866	*Sesbania aculeata* Auct.	4	5	2	5
QA 874	*Aeschynome indica* Austral.	1	3	2	3
Trifolium cross-inoculation grouping					
QA 859	*Trifolium subterraneum* L.	0	3	0.5	2.5
TA 1	,,	0	2	0.5	3 †
RTR 151	*T. repens* L.	1	1	2	3
RTM 755	*T. polymorphum* L.	0	0	1	2
Medic cross-inoculation grouping					
TC 4	*Medicago sativa* L.	0	5 †	1.5	4.0 †
QA 383	,,	1	2 †	1	4 †
Pea cross-inoculation grouping					
QA 384	*Pisum sativum* L.	1	2	3	5
Bean cross-inoculation grouping					
RPV 790	*Phaseolus vulgaris* L.	0	0 †	1.5	2

* measured from the surface of the seed to the edge of the zone of inhibition.

† Stimulation of growth outside the zone of inhibition.

gives data on the radius of complete and partial inhibition zones with *C. pubescens* and clover.

While not being restricted to any particular groups of Rhizobium strains, the degree of inhibition varied markedly with Rhizobium strain and legume species. Generally seed diffusate from clover was more active than that from *C. pubescens*.

In some instances zones of stimulation occurred beyond the inhibition zones.

Toxicity toward other organisms

Eighteen bacteria and one yeast, including rhizosphere, soil,

TABLE 2

Inhibition of organisms other than Rhizobium by seed diffusates of *C. pubescens* and subterranean clover

Organism	Habitat	Probable identity	Gram reaction	Radius of inhibition * mm	
				C. pubescens	Subterranean clover
C 13	Tomato seed coat	*Bacillus* sp.	+	4	5
C 30	,,	,,	+	5	8
& R 1	Subterranean clover root	*Arthrobacter* sp.	−	3	3
& R 9 †	,,	P.X.F.E. group	−	1	1
& R 13 †	,,	X.F. group	−	3	3
& R 37 †	,,	P.A. group	−	2	2
& R 15	,,	*Arthrobacter* sp.	+	2	3
TT 11A	Tomato root tip	yeast	+	1.5	0.5
T 2A	,,	unidentified	−	0	0.5
PP 74	Pea root	unidentified	−	0.5	0.5
8 C_1	*Pinus radiata* root	*Bacillus* sp.	+	7	7
chromobacter sp.	Soil	*Achromobacter* sp.	−	0.5	0.5
grobacterium diobacter	,,	*Agrobacterium radiobacter* (N.C.I.B. 8149 DSIR Gt. Britain)	−	0	0.5
rthrobacter citreus	,,	*Arthrobacter citreus*	±	5	7
lavobacterium sp.	,,	*Flavobacterium* sp.	−	0	0
ocardia rubra	,,	*Nocardia rubra*	−	0	0
eudomonas medi-ginis	Plant pathogen	*Pseudomonas medicaginis*	−	3	5
. syringae	,,	*Ps. syringae*	−	0.5	1
cherichia coli	Intestinal	*Escherichia coli*	−	0	0

* Distance from edge of seed to outer edge of zone of complete inhibition.

† Organisms and identity supplied by Joan I. Sperber and A. D. Rovira. (Sperber and Rovira[17])

P.X.F.E. = Pseudomonas-Xanthomonas-Flavobacterium-Erwinia.
X.F. = Xanthomonas-Flavobacterium.
P.A. = Pseudomonas-Achromobacter.

plant-pathogenic and enteric organisms, were tested with lucerne, subterranean clover and *C. pubescens* seed using the agar-plate assay. Lucerne seed diffusate again produced little inhibition. Radii of zones of complete inhibition by *C. pubescens* and clover (Table 2) showed seed diffusates from the two species to be active against a wide range of organisms but generally clover diffusate was the more active. In some instances non-germinating *C. pubescens* seed produced a slight inhibition. The extent of inhibition varied with the bacterium and some organisms, *e.g. Flavobacterium* sp. and *Nocardia rubra* were not affected. Inhibition of gram-positive bacteria was generally greater than that of gram negative although some of these also (*e.g. Ps. medicaginis*) were very strongly inhibited.

Site of production of toxicity

The testa was dissected from surface-sterilized *C. pubescens* and clover seed without injury to the remainder of the seed and inhibition from intact seed compared with that produced by the testa and seed without testa. The test micro-organisms were *Arthrobacter citreus* and Rhizobium strains QA 616 (from *C. pubescens*) and RPV 790 (from *Phaseolus vulgaris*). The results (Table 3 and Plate 4) showed that almost all antibacterial activity arose from the seed coat.

TABLE 3

Site of production of toxin in *C. pubescens* and subterranean clover			
Seed and test organism	Radius of inhibition *, mm		
	Whole seed	Testa	Seed without testa
C. pubescens			
Arthrobacter citreus	2	1.5	0
Rhizobium sp. QA 616	2.5	1.5	0
R. phaseoli RPV 790	0.5	0.5	0
Subterranean clover			
Arthrobacter citreus	3	2	1
Rhizobium sp. QA 616	1.5	1.5	0
R. phaseoli RPV 790	2	1.5	slight

* Radius of complete inhibition after 2 days on YM agar.

Multiplication of organisms around seed in sand and soil

In order to examine the relationship between antibiotic activity indicated by the agar-plate assay and multiplication of organisms

Plate 1. Inhibition of *R. leguminosarum* (left) and of *R. phaseoli* (right) by diffusates from subterranean clover seed (Seed removed before photographing).

Plate 2. Inhibitory effect of diffusates from germinating *C. pubescens* seeds on *Rhizobium sp.* isolated from *C. pubescens*.

Plate 3. Inhibition of *Arthrobacter citreus* (left) and *Bacillus sp.* (right) by diffusates from subterranean clover.

Plate 4. Non-inhibition of *R. phaseoli* by subterranean clover seeds devoid of testa (left), inhibition by diffusates from testa alone (centre) and from intact seeds (right).

around seeds in a more natural environment, inoculated subterranean-clover seeds were sown in heat-sterilized, washed Waikerie river sand and Urrbrae loam (a red brown earth) moistened to field capacity with plant nutrient solution (Hoagland and Arnon [22]). Glass beads of approximately the same size were treated identically as controls. Seeds and beads were surface sterilized, and planted in the sand and soil after inoculation with approximately 1000 organisms per seed or bead with either the subterranean clover *Rhizobium* RTR 151, subterranean-clover rhizosphere isolate S and R 13 (belonging to the *Xanthomonas-Flavobacterium* grouping of Sperber and Rovira [17]) or the soil and rhizosphere organism *Agrobacterium radiobacter*. Viable counts were then made from each of 10 seeds or beads in each treatment immediately and after 24 hours at 25°C. The results (Table 4) are expressed as a ratio of the mean final population to the mean initial population – referred to as the "proliferation factor".

TABLE 4

Growth of micro-organisms on subterranean clover seed and glass beads

Organism	Radius of inhibition, mm	Proliferation factor *			
		Sand		Soil	
		Seed	Beads	Seed	Beads
S&R 13 (Xanthomonas-Flavobacterium)	3	0.43	22.8	0.24	9.03
RTR 151 (*Rhizobium trifolii*)	2	19.9	117	0.86	1.1
Agrobacterium radiobacter	0.5	194	49	114	24.5

* Proliferation factor $= \frac{\text{Mean final population/seed or bead}}{\text{Mean initial population/seed or bead}}$.

The rhizosphere isolate S and R 13 (moderately susceptible by the agar plate assay) showed an absolute decline in seed population in both sand and soil whereas multiplication took place freely around glass beads. The *Rhizobium trifolii* strain RTR 151 while multiplying on seed in sand did so to a much less extent than on glass beads and an inhibitory effect of seed diffusate can be implied. In soil a decline in population occurred around seed whereas a slight rise occurred around beads. It is apparent that seed was toxic to the

Rhizobium. The low multiplication on beads may be due to a suppression of *Rhizobium* by the soil itself or products of heat sterilization or to rapid diffusion of organisms away from the bead (or seed) under the test conditions. *Ag. radiobacter* was not inhibited by seed diffusates under the test conditions and was in fact greatly stimulated by them.

Counts of two Rhizobium strains from *C. pubescens* inoculated onto *C. pubescens* seed in sand and soil showed a considerably lower population around swelling seed after 24 hours than around hard seed. The agar-plate tests had shown inhibition of rhizobia only from germinating seeds.

Although only a few tests have been made it seems that effects obtained in sand and soil parallel those on agar and the multiplication of many gram-positive and gram-negative organisms in sand and soil will be restricted by seed-coat diffusates.

DISCUSSION

The results are of particular interest in that a marked antibiotic activity is shown by legume seeds against their symbiont, Rhizobium and also against a range of other gram-negative organisms. The demonstration of antibiotic activity toward a wide range of rhizobia extends the observation of Thompson [19] from one Rhizobium strain to several. In the present studies gram-positive or gram-variable bacteria were more susceptible to the seed diffusates than gram negative. A greater sensitivity of gram-positive organisms toward wide spectrum bactericides has been shown frequently (Bartholomew and Mittwer [3]). Since no bioassays have been performed on different fractions of the seed diffusates from *C. pubescens* and subterranean clover it is not known whether the same substance is active toward both gram-positive and -negative organisms.

Are toxic seed diffusates likely to be important in inoculation of legume seeds with Rhizobium? The data of Vincent [21] and Thompson [19] indicate the marked deleterious effect of diffusates which may occur when inoculum is applied to subterranean clover in liquid form. Diffusion of substances from the seed coat of subterranean clover occurs very rapidly and it may be expected that any treatment moistening the seed coat will extract some toxin.

This may be part of the reason for the poor survival of *Rhizobium* when seed is inoculated before lime pelleting (Vincent [21]). Diffusion of substances from the seed coat of *C. pubescens* appears to be appreciably slower than from subterranean clover and in this case treatment with liquid inoculum may have a lesser effect. The investigations indicate also that the severity of seed-coat toxicity to rhizobia may be lessened by proper selection of Rhizobium strains.

These studies have shown also that seed-coat toxicity to rhizobia can influence rhizobial proliferation around seed planted in sand or soil. In some instances populations may decline, and in others multiplication may be restricted. The consequences of this cannot accurately be forecast because of the complex and dynamic status of soil. In most soils under suitable planting conditions good nodulation usually follows liquid inoculation of seed. The presence of toxic seed diffusates by itself may not have major economic effects under most conditions but where other factors are reducing rhizobial multiplication, toxic seed diffusates may then make multiplication around the seed more unlikely. Many factors are likely to determine the interaction between seed diffusate and micro organisms in the field. Both the rate of diffusion of toxic substances from the seed, and the concentration around the seed will be influenced markedly by soil moisture, which may also affect the ability of Rhizobium strains to move away from the seed. The rate of decomposition of the diffusates by other soil organisms and the effect of these on legume bacteria is unknown. It is conceivable that resistance to seed diffusate may partially determine success of a Rhizobium strain in a mixture and be a factor in the incursive property of rhizobia described by Harris [9].

Seed diffusates have a marked effect on the composition of the seed-coat microflora during germination. Rovira [16] showed a selective multiplication of organisms on the seed coat of germinating tomatoes. The present investigations show that in considering the composition of the spermatosphere not only must a response to excreted nutrients (Stille [18], Fries and Forsman [7], and Picci [15]) be considered but also selective inhibition of microorganisms by toxic exudates. The presence of seed-coat toxins would place the gram-positive bacteria of the seed coat (Wallace and Lochhead [22]) at a greater disadvantage than gram-negative organisms during seed germination and this may be expected to influence the composition of the early rhizosphere population.

SUMMARY

Seed coat diffusates from the legumes *Centrosema pubescens* and subterranean clover were found to contain a water-soluble, thermostable, antibiotic inhibiting a wide range of rhizobia and other gram-negative organisms as well as gram-positive organisms. Lucerne seed diffusate showed little activity. The extent of inhibition varied with the micro-organism. Diffusate from subterranean clover was generally more active than from *C. pubescens*.

It was shown that seed diffusates can have a depressive or a stimulative effect on multiplication of organisms around seeds germinating in sand and soil.

The significance of the results, particularly with respect to inoculation with Rhizobium is discussed.

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THE EFFECTS OF MICRO-ORGANISMS ON PLANT GROWTH

I. DEVELOPMENT OF ROOTS AND ROOT HAIRS IN SAND AND AGAR

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INTRODUCTION

Hiltner [16] assigned the term "rhizosphere" to that zone of soil influenced by the root and found greater numbers of micro-organisms in this region than in soil beyond the rhizosphere. He suggested that this intense development of micro-organisms could influence plant growth. The reviews by Starkey [36], Katznelson ,Lochhead, and Timonin [20], Clark [9] and Starkey [37] show that a great deal is known about the effects of plant roots on soil micro-organisms but little is known of the effects of rhizosphere organisms on plant development apart from Rhizobium, mycorrhizae and pathogens.

Studies of non-invading rhizosphere organisms on laboratory media have formed a basis for much speculation on their roles in the inorganic nutrition of plants. In only very few instances have the speculations been supported by inoculation of plants under controlled conditions. For example, while a number of workers (Gerretsen [13], Sperber [35], Katznelson and Bose [19], Louw and Webley [23]) have now studied solubilization of poorly soluble phosphorus sources by rhizosphere micro-organisms, apparently only Gerretsen [13], Pikovskaya [32] and Krasilnikov and Kotelev [22] have shown increased uptake of phosphate from poorly soluble sources following addition of rhizosphere organisms to plants growing in sterile soil. Askinazi [4] considered that the work carried out on phosphatic bacterial fertilizers in the U.S.S.R. over the previous ten years had not yielded significant results. Timonin [41] has related laboratory studies on oxidation of manganese by rhizosphere micro-organisms to availability of manganese to growing plants. He found that partial soil sterilization reduced the manganese oxidizing bacteria in the rhizosphere of a susceptible variety of oats with consequent

reduction in manganese-deficiency symptoms. Bromfield [7] recorded greater manganese content in oats grown aseptically than in those grown in the presence of micro-organisms.

There have been many claims made for nitrogen fixation by non-symbiotic organisms in the rhizosphere. First advanced by Caron [8], this thesis received only sporadic recognition until some twenty years ago when the practice of seed inoculation with species of *Azotobacter* became established in U.S.S.R. – "Azotobacterin". While being critical of much of the experimentation Jensen [18] and Allison [3] referred to some instances where a real stimulation of plant growth is beyond doubt. Both considered that the plant growth increases would be more adequately explained by phenomena other than nitrogen fixation, such as antagonism to harmful micro-organisms or production of organic growth stimulants. Parker [29] found that a grass sward fixed 165 pounds of nitrogen per acre in three years and while rhizosphere organisms could have been active in this fixation it was difficult to discriminate between these and saprophytes decomposing sloughed root material. Stevenson [38] used isotopic nitrogen to show marked nitrogen fixation by non-nodulated plants and points out the possibility that organisms associated with the roots may be responsible – especially mycorrhizal associations.

Most reports of controlled experimentation with non-infecting micro-organisms claim plant stimulation while a few report reduction in plant growth. Swaby [40] incorporated a range of organic materials in sand and grew lucerne and phalaris under sterile and non-sterile conditions. He found that in some cases presence of micro-organisms greatly stimulated growth (up to 500 per cent increase) while in others a marked decrease occurred (up to 92 per cent decrease) with the two test plants often responding differently. In treatments with no added organic matter non-sterile lucerne plants were almost twice as large as sterile. Phalaris grown under non-sterile conditions was also larger than that grown under sterile conditions. Pantos [28] found that all of eight wheat rhizosphere bacteria stimulated both top and root growth of wheat in sterile sand culture. One organism, *Pseudomonas radiobacter* (*Agrobacterium radiobacter*) produced a 65 per cent increase over the control. These stimulations agree with the results of Russian workers mentioned by Pantos. Cooper [10] reported claims of S. F. Lazarev for "Azotobacterin" promoting development of cotton roots even though yield was not affected, and other Russian claims of stimulation or inhibition of germination of sugar beet by rhizosphere organisms. Kerr [21] showed that the plant pathogen *Sclerotinia homeocarpa* growing in the rhizosphere, although not infecting the plant, produced substances retarding germination and seedling growth of tomato, beet, radish, lettuce, subterranean clover, and wheat. An apparent inhibition of maize roots in agar culture by two *Penicillium* species reported by Stolp [39] was due to the inoculating medium rather than the fungi. It has been shown by Gottlieb [14], Stolp [39] and others that filtrates of media in which micro-organisms had grown inhibited root growth but this is no indication of any similar action by the same organism growing in the rhizosphere.

Rhizosphere organisms have been recorded as influencing root morphology and development. The best known of these are curling of legume root hairs prior to infection by Rhizobium – a non-specific curling which can be induced by several soil organisms (Wilson [43]) – and forking of short roots of *Pinus* species which can occur prior to mycorrhizal infection (Levisohn [24]). Although in both cases indoleacetic acid may be involved, data of Ekdahl [11] and Barnes and Naylor [5] show that this is not the only substance capable of producing such changes. Stolp [39] found that a culture of the *Bacillus circulans* group and *Bacillus fusiformis* caused frequent forklike ramifications of root hairs of maize in agar culture which he regarded as an artefact induced by reduction in agar consistency by bacterial proliferation.

Rovira and Bowen [34] showed that the growth of roots and root hairs of subterranean clover grown in sand and agar was reduced by the presence of a general rhizosphere microflora. This phenomenon has been studied in greater detail for a range of plant species and the results are reported in this paper.

MATERIALS AND METHODS

(a) *Plants*

Test plants were: the Bacchus Marsh variety of subterranean clover (*Trifolium subterraneum* L.), tomato (*Lycopersicon esculentum* Mill.), phalaris (*Phalaris tuberosa* L.) and radiata pine (*Pinus radiata* D. Don).

(b) *Planting procedure*

Seed of a uniform size was surface sterilized by shaking in a 7% aqueous solution of calcium hypochlorite for 30 to 60 minutes according to the seed, followed by two washings with sterile distilled water. The seeds were germinated on a glucose–yeast–peptone agar (Rovira [33]) at 25°C. Uncontaminated seeds at a uniform stage of root emergence were transferred aseptically to large test tubes containing sand or agar.

(c) *Growth substrates*

Sand culture was in 3 × 20 cm test tubes with 50 g (5-cm depth) of washed Waikerie river sand moistened to field capacity with plant nutrient solution (Hoagland and Arnon [15]) and steam sterilized at 130°C for 1 hour. Initial studies in agar employed 25 ml of plant nutrient solution solidified with 1% agar and sterilized at 120°C for 30 minutes. Three plants per tube and four replications were used. For later studies on progressive root elongation in agar 1.5 × 25 cm tubes were used with 15-cm depth of plant nutrient agar (referred to as "agar deeps") with one plant per tube. Twelve replications were employed unless otherwise stated. Unless specifically mentioned all plants were grown in a glasshouse fitted with supplementary heating and cooling.

(d) *Inoculation*

Tubes in non-sterile treatments were inoculated at planting with 0.1 ml of 10% suspension of soil in sterile water. Sterile treatment tubes received 0.1 ml of an autoclaved preparation of the suspension. Before harvesting, controls were examined for sterility. The soils used for preparation of inocula were:

Urrbrae red-brown earth (*URBE*) – under permanent lucerne, rye grass, subterranean clover, and phalaris pasture

Second Valley laterite (*SVL*) – under native vegetation

Mount Compass sand (*MCS*) – under native vegetation

Claremont black earth (*CBE*) – a clay soil under permanent pasture.

(e) *Assessment of results*

(i) Root growth and development. Details of root length and root patterns were recorded diagramatically and analysed with respect to total root length, length of primary roots, number, concentration and length of secondary roots.

(ii) Root hairs. Relative numbers of root hairs, length, and positions of development of root hairs were recorded following observations with a binocular dissecting microscope. ($\times 45$ or $\times 20$).

RESULTS

a. ROOT GROWTH

(i) *Effects of micro-organisms from Urrbrae red-brown earth*

The test species were sown in sand and in agar and inoculated with sterile and unsterile URBE soil suspensions. Harvests of subterranean clover, tomato and phalaris were made after six weeks and of radiata pine after six months. Table 1 summarises the root analyses and Figures 1, 2 and 3 show root patterns of typical plants in sand and agar.

Total root length (primary plus secondary roots) were significantly reduced in almost every instance by the presence of micro-organisms.

The reduction in primary-root length due to micro-organisms proved to be statistically significant for all plant species grown in sand and for clover, tomato, and phalaris grown in agar. This was in spite of considerable variation between replicates. The reduction in mean primary root length of pine in agar due to the micro-organisms was not significant.

There were also distinct tendencies for reduction of length of secondary roots. Although the total numbers of secondary roots

TABLE 1

Root growth of test plants under sterile and non-sterile conditions in sand and agar.

Test plant	Subterranean clover		Tomato		Phalaris		Radiata pine	
Condition	Sterile	Non-sterile	Sterile	Non-sterile	Sterile	Non-sterile	Sterile	Non-sterile
a. Sand								
Total root length (cm)	17.0	11.3*	28.6	16.5*	47.9	35.7	14.7	9.9*
Primary-root length (cm)	8.3	5.7**	8.0	3.6***	24.4	14.6**	7.1	5.5*
Secondary-root length (cm)	8.5	5.6	15.4	10.0	21.5	15.0**	7.1	4.3
No. primary roots	1	1	1	1	5.7	4.6	1	1
No. secondary roots on 1st primary	8.1	6.5	17.4	12.0*	32.3	24.3**	16.6	7.8**
Mean length secondary root (cm)	1.14	1.18	0.91	0.87	0.67	0.61	0.40	0.32
Secondary roots/cm primary root	0.99	1.07	2.30	3.50**	2.37	2.95	2.41	1.73
Plant top weight (mg, oven dry)	11.3	9.3					53	28***
b. Agar								
Total root length (cm)	18.9	12.3*	44.8	26.3**	44.7	25.7*	17.0	13.9
Primary-root length (cm)	12.4	8.3***	13.6	5.0***	35.3	17.4***	15.7	12.6
Secondary-root length (cm)	6.5	4.0	30.8	11.7*	9.5	7.5		
No. primary roots	1	1	1	1	6.3	5.0**	1	1
No. secondary roots on 1st primary	4.7	3.2	15.7	5.7*	35.6	11.2*		
Mean length secondary root (cm)	2.30	1.03	1.95	1.91	0.26	0.57*		
Secondary roots/cm primary root	0.40	0.46	1.17	1.96	1.15	1.22		
Plant top weight (mg, oven dry)	8.2	6.4					59	61

* Sterile and non-sterile differ significantly at 5% level.
** ,, ,, ,, ,, ,, ,, ,, 1% level.
*** ,, ,, ,, ,, ,, ,, , 0.1% level.

were higher with sterile plants this was probably the result of greater development of the primary root. The concentration of secondary roots on the primary was slightly greater with unsterile plants than with sterile.

Except for radiata pine growing in sand – where sterile plants were significantly larger – there was little difference in growth between sterile and non-sterile plants.

Fig. 1. Root diagrams of typical tomato and subterranean clover grown in sterile and non-sterile sand and agar.

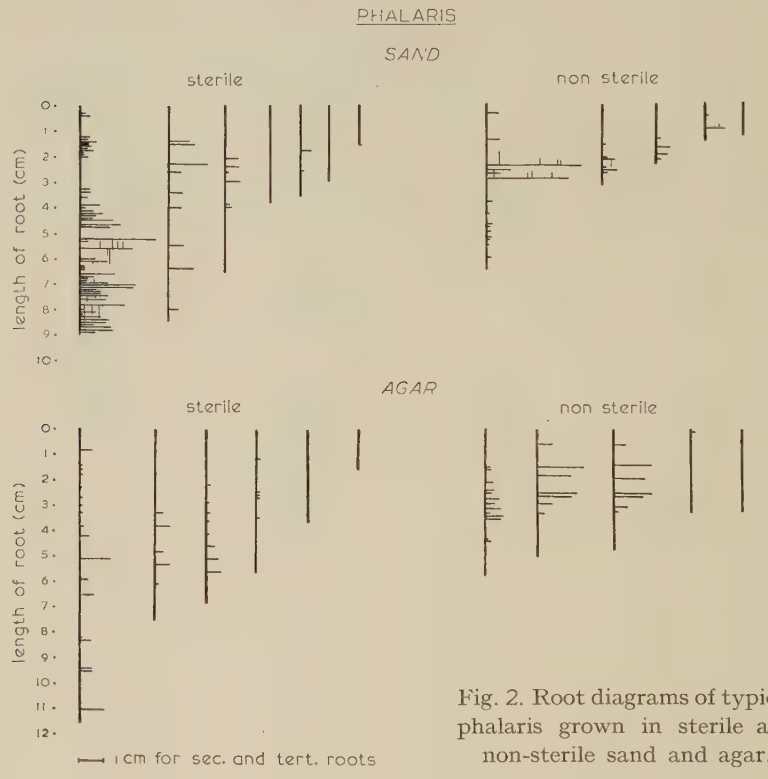


Fig. 2. Root diagrams of typical phalaris grown in sterile and non-sterile sand and agar.

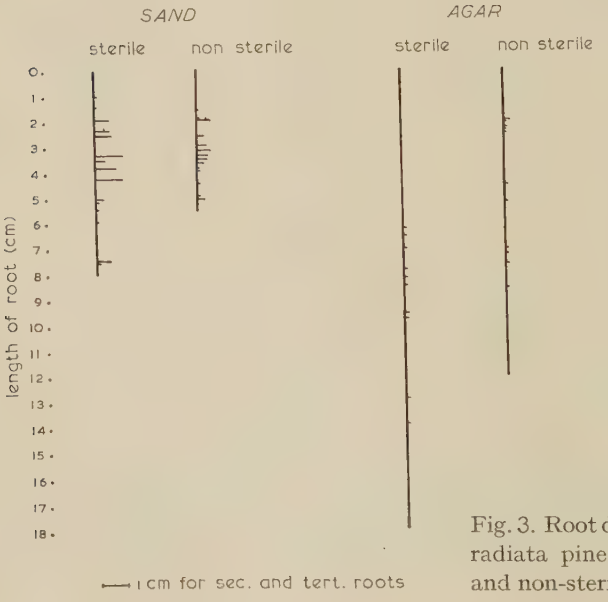


Fig. 3. Root diagrams of typical radiata pine grown in sterile and non-sterile sand and agar.

(ii) *Effects of different soil inocula*

Subterranean clover plants in agar deeps were inoculated with non-sterile soil suspensions from *URBE*, *SVL*, *MCS* and *CBE* and with sterile *URBE* soil suspension. Ten plants were grown in each treatment and harvested after four weeks.

The results (Table 2) showed root stunting with organisms from all four soils with variations in the degree of stunting with the different soils.

TABLE 2

Root stunting of subterranean clover in agar with inocula from different soils

Inoculum	Range	Mean	Significance *
a. *Total root length*			
Sterile	20.5–30.7 cm	23.6 cm	Significantly greater than all other treatments (5%)* Mount Compass sand and Urrbrae red-brown earth (1%)
Mount Compass sand .	16.4–23.8	19.2	No significant difference.
Claremont black earth	14.2–27.4	19.3	
Second Valley laterite	5.5–28.4	16.7	
Urrbrae red-brown earth	5.3–26.3	14.9	
b. *Primary-root length*			
Sterile	15.5–21.0 cm	18.4 cm	Significantly greater than all other treatments (1%) *
Mount Compass sand .	13.5–17.2	15.8	Significantly greater than Urrbrae red-brown earth (5%).
Claremont black earth	13.5–20.0	15.3	No significant difference
Second Valley laterite .	5.5–18.0	13.3	
Urrbrae red-brown earth	3.5–17.0	12.2	

* Figures in parenthesis indicate level of significance.

The generality of root stunting by organisms from the four soils was confirmed in sand culture.

(iii) *Abundance of root-stunting organisms in Urrbrae red-brown earth*

Subterranean clover planted in agar deeps was inoculated with 0.1 ml of successive tenfold dilutions of *URBE*. The mean daily increments in root growth over 20 days are given in Table 3. In this experiment six replicates were employed.

Root stunting was present at the 1 in 10 dilution and in some plants in the 1 in 100 dilution but absent in higher dilutions whereas microbial proliferation occurred to 1 in 10,000,000. It is inferred

TABLE 3

Abundance of root-stunting micro-organisms in Urrbrae red-brown earth				
Inoculum	No. plants with micro-organisms	Daily root elongation (mm)		Difference from controls
		Range *	Mean	
Nil	0	3.9–4.8	4.4	
Sterile 10^{-1} dilution	0	3.6–5.1	4.3	
Non-sterile 10^{-1} dilution	6	1.5–2.8	2.4	Signif. at 0.1% level
,, 10^{-2} ,,	6	1.5–4.7	3.5	Not significant
,, 10^{-3} ,,	6	4.3–5.4	4.7	,,
,, 10^{-4} ,,	6	3.3–4.5	3.8	,,
,, 10^{-5} ,,	5	3.8–4.8	4.1	,,
,, 10^{-6} ,,	3	3.9–4.7	4.3	,,
,, 10^{-7} ,,	3	4.1–4.6	4.3	,,

* Mean of 6 plants.

from this that *URBE* root stunting organisms are present in concentrations of at least a thousand per gram of soil and that specific micro-organisms are involved.

(iv) *Time of onset of root stunting*

Subterranean clover and tomato in agar deeps and inoculated with *URBE* suspensions were grown in a controlled growth room

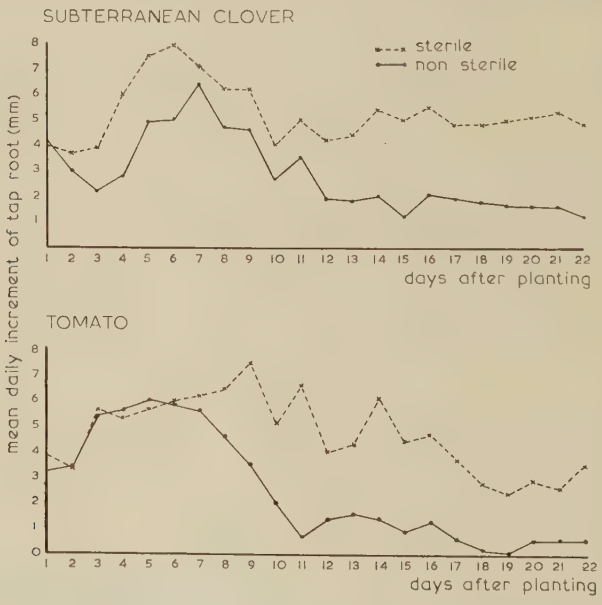


Fig. 4. Daily root increment of subterranean clover and tomato in sterile and non-sterile agar.

and daily primary-root elongation recorded for 22 days. The growth room gave a 12-hour light period of 1,000 foot candles with a temperature of 18°C and a 12-hour dark period with a temperature of 15°C. Daily primary-root elongations are presented in Figure 4, and Plates 1 and 2.

In this study great variability between plants occurred and although sterile clover showed a tendency for greater daily root elongation than unsterile plants from the time of planting, differences between the two treatments were not significant until the

Plate 1. Daily root elongation of subterranean clover in sterile (left) and non-sterile (right) agar.

fifth day. Differences were not significant with tomato until the ninth day although daily root elongation with some plants was declining by the fifth day. With both species the increase of root elongation at the 6-7 day period coincided with cotyledon emergence and opening.

Progressive root-elongation measurements revealed a particularly high variation among non-sterile plants. In some instances stimulation occurred, followed by stunting (Plate 3). However it has not

Plate 2. Daily root elongation of tomato in sterile (left) and non-sterile (right) agar.

been ascertained whether stimulation coincided with proliferation of particular micro-organisms at that time.

Studies with progressive harvesting of subterranean clover, tomato and phalaris growing in sand showed that root-length differences are present at 3 to 4 weeks. Slower root growth in sand and plant variability made an earlier demonstration difficult without greatly increased replication.

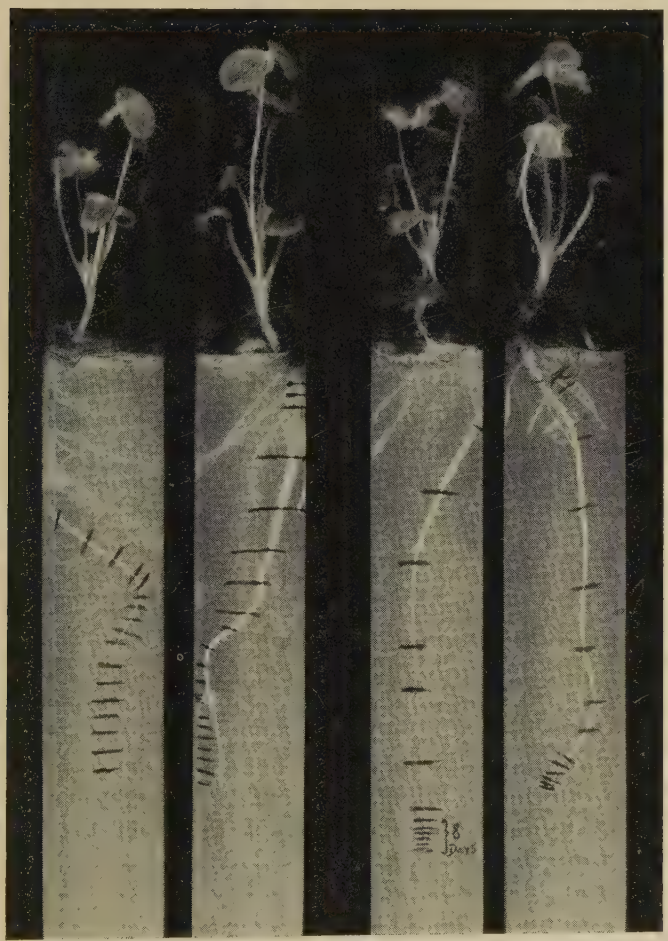


Plate 3. Daily root elongation of subterranean clover in non-sterile agar. Plants on right show early root growth stimulation followed by stunting.

(v) *Factors responsible for root stunting*

Preliminary studies have been made on the effects of aeration on root stunting and also on the transfer of the effect.

a. Aeration of roots. Subterranean clover and tomato were grown on long agar slopes with the roots extending along or just below the agar surface. When these were inoculated with sterile and non-sterile *URBE* soil suspensions, there was a highly significant reduction in primary root length of non-sterile roots (Table 4). The degree of stunting due to micro-organisms was similar to that obtained in agar deeps. (see Table 2).

TABLE 4

Root growth of subterranean clover and tomato on agar slopes.

	Subterranean clover		Tomato	
	Sterile	Non-sterile	Sterile	Non-sterile
Mean length of primary root (cm) . .	24.7	9.4***	14.6	11.0***
Mean total root length (cm)	78.1	48.3**	53.3	55.0
Mean No. secondary roots	27.8	17.5*	27.0	26.5
Mean No. secondary roots/cm primary root	1.12	1.97**	1.84	2.41*

* Differences between sterile and non-sterile significant at 5% level.
** ,, ,, ,, ,, ,, ,, ,, 1% level
*** ,, ,, ,, ,, ,, ,, ,, 0.1% level.

With tomato better secondary root development occurred in the non-sterile series which resulted in no significant differences in total root lenghts. Mean numbers of secondary roots per centimetre of primary root were significantly greater with non-sterile plants. As in earlier experiments variability was greater in the non-sterile series.

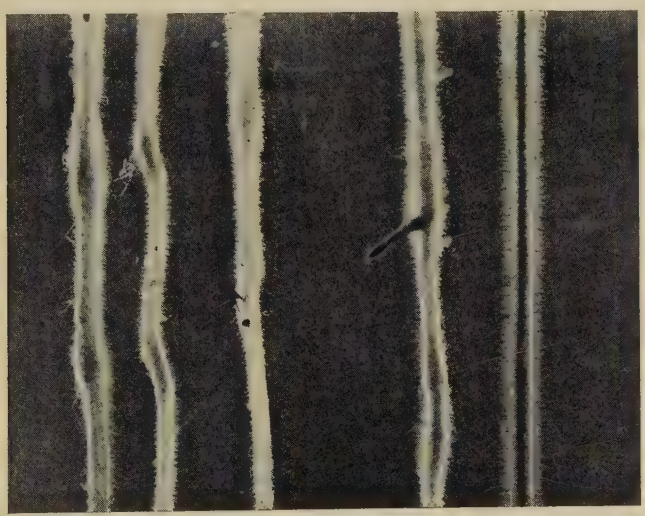
b. Transfer of effect. Agar deeps in which subterranean clover inoculated with suspensions from *URBE* had been grown for 4 weeks with marked root inhibition in non-sterile tubes were resterilized by steam at 100°C for 1 hour on each of two successive days. On replanting without re-inoculation root inhibition did not occur.

b. ROOT-HAIR DEVELOPMENT

(i) *Root-hair effects exerted by micro-organisms*

An examination of six weeks old subterranean clover grown in sand inoculated with Urrbrae red-brown earth suspensions revealed

marked root-hair differences between sterile and non-sterile plants. There was a uniformly profuse development of long root hairs over the roots of sterile control plants. However in non-sterile treatments root hairs at the crown were reduced in length and concentration; in the mid-root region root hairs were very sparse, poorly developed and absent from this region to the tip (approximately 5 cm). Root-hair development on non-sterile lateral roots was very poor. In agar, root-hair production in non-sterile treatments was quite variable and greatly reduced in comparison with sterile plants (see Plate 4).



a b c d e

Plate 4. Root hair development of subterranean clover growing in agar.
(a) Mount Compass sand inoculum.
(b) Claremont black earth inoculum.
(c) Urrbrae red-brown earth inoculum.
(d) Second Valley laterite inoculum.
(e) Sterile Urrbrae red-brown earth inoculum.

With tomato and phalaris micro-organisms caused a slight root-hair reduction in sand, but not in agar. Radiata pine developed very few root hairs in either substrate under the test conditions.

(ii) *Effects of the different soil inocula*

Subterranean clover growing in sand and agar inoculated with non-sterile suspensions of four soils *viz URBE, MCS, SVL,* and

CBE and with sterile *URBE* suspension as the control showed marked reduction in the concentration and extent of root hairs along the primary root and in root-hair length, except for *MCS* inoculum in sand with which less root hair suppression was observed. The results given in Table 5 and Figure 5 and illustrated in Plate 4 show the effects of micro-organisms on the root-hair development with plants grown in agar.

TABLE 5

Root-hair suppression by inocula from different soils.

Inoculum	Range	Mean	Significantly differing from
a. *Distance between root tip and root-hair zone*			
Sterile	0.1–0.3 cm	0.2 cm	*MCS, SVL, CBE* (0.1%)*, *URBE* (1%).
Mount Compass sand (*MCS*)	0.2–3.0	1.5	*CBE* (0.1%)
Urrbrae red-brown earth (*URBE*)	0.3–3.5	1.9	*CBE* (1%)
Second Valley laterite (*SVL*)	0.7–4.0	2.1	*CBE* (0.1%)
Claremont black earth (*CBE*)	2.0–6.0	4.6	
b. *Length of root with hairs greater than 120μ long*			
Sterile	4.0–13.0 cm	8.2 cm	*CBE, URBE* (0.1%) * *MCS, SVL* (1%).
Mount Compass sand (*MCS*)	0 – 8.0	5.0	*URBE* (5%)
Claremont black earth (*CBE*)	0 – 6.0	3.5	
Second Valley laterite (*SVL*)	0 – 8.5	3.8	
Urrbrae red-brown earth (*URBE*)	0 – 4.0	2.3	
c *Maximum length of root hairs*			
Sterile	150–300μ	200μ	*SVL* (0.1%) *, *MCS, URBE, CBS* (1%).
Mount Compass sand (*MCS*)	90–200	140	
Urrbrae red-brown earth (*URBE*)	120–300	140	
Claremont black earth (*CBE*)	60–180	130	
Second Valley laterite (*SVL*)	90–180	120	

* Figures in parentheses indicate level of significance.

While marked inhibition of root-hair numbers and length occurred in agar with all non-sterile soil inocula, the root-hair development by plants inoculated with *MCS* suspension was significantly greater than with inocula from other soils. It is thought that particular organisms are involved in the effect and their differential proliferation along the root may account for the uneven distribution of root hairs over the root. Further, it is apparent that not only was root-hair initiation delayed and suppressed, but also

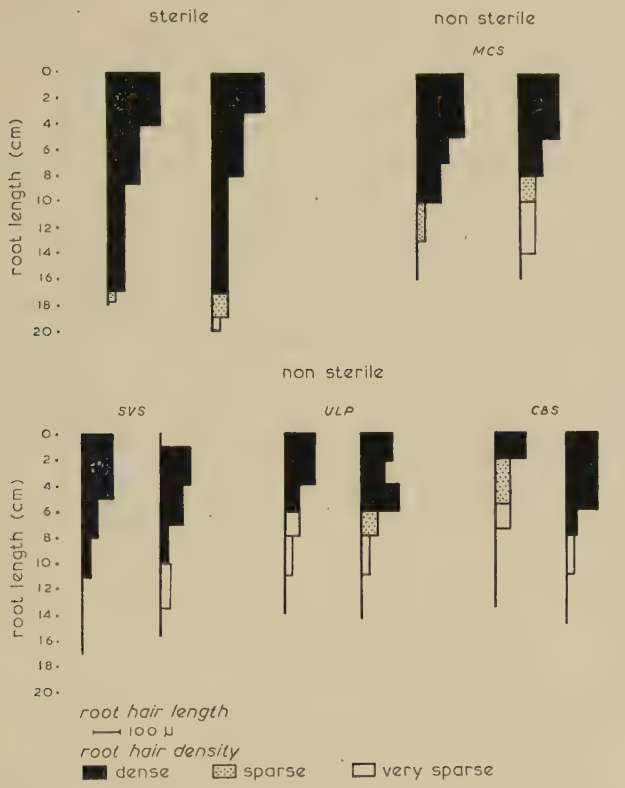


Fig. 5. Density and length of root hairs on subterranean clover in sterile and non-sterile agar.

Inoculation: – *MCS; CBE; SVL;* and *URBE*. For abbrevations see Table 5.

after initiation, retardation of root hair growth occurred. It is inferred from these observations that the microflora exerts a direct effect on root-hair development rather than destroying existing root hairs by microbial cellulases and pectinases.

(iii) *Abundance of root-hair-inhibiting organisms in Urrbrae red-brown earth*

Subterranean clover growing in sand was inoculated with tenfold dilutions of *URBE* suspension and examined four weeks later. Root-hair suppression occurred in all dilutions to 1 in 100,000 but at 1 in 1,000,000 not all plants showed the effect. No plants showed the effect above this dilution although organisms were present on the roots of plants inoculated with the 1 in 10,000,000 dilution.

It may be concluded that root hair inhibition is caused by particular micro-organisms, which numbered at least 100 million per gram of *URBE* at the time of sampling.

(iv) *Onset of root-hair inhibition*

Subterranean clover growing in sand in a controlled growth room (see Part a) and inoculated with sterile and unsterile *URBE* soil suspensions were harvested at 1, 3, 7, 12, and 19 days. At 3 days root hairs on sterile plants were distributed uniformly over the roots to within 1 to 2 mm. from the tip whereas those on non-sterile roots were usually shorter and irregularly distributed. Root-hair inhibition by micro-organisms became more obvious as the plants developed. (Fig. 6). This also supports the conclusion that with seedlings there is a definite inhibition and retardation of root hairs by micro-organisms rather than a microbial digestion of previously formed root hairs.

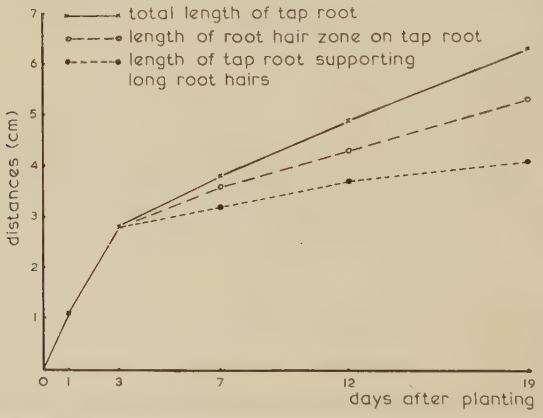


Fig. 6. Progressive development of root hairs of subterranean clover growing in non-sterile sand.

(v) *Factors responsible*

During preliminary studies with clover attempts were made to transmit the effects in the absence of micro-organisms. Subterranean clover was grown in plant nutrient solution for 6 weeks and root-hair inhibition by micro-organisms observed. One half of this solution was then sterilized by heating to 100°C for 1 hour on each of 3 successive days and the other half, by passing through an

"Equip" D-9 filter. Control solution in which subterranean clover had grown aseptically was treated similarly. A slight transmission of root-hair inhibition on clover planted in these solutions occurred only in the filtered, previously non-sterile solution.

DISCUSSION

The marked suppression of root length by micro-organisms growing in association with the four test plants indicates the phenomenon to be of wide occurrence. Further, the more detailed study with subterranean clover shows that root-growth suppression may occur with organisms from a range of quite different soil types. This root inhibition by a general soil inoculum is in marked contrast to the results of others (*e.g.* Swaby [40], Gerretsen [13]). However the improvement of growth by micro-organisms found by Gerretsen was due specifically to their rendering phosphate available. Rovira [33] has shown the effects of temperature and light intensity variation on the nature of root exudates and if the present results are to be reconciled with those of Swaby, it may be that slight environmental differences are sufficient to change the root exudate, rhizosphere composition and hence the plant—micro-organisms interaction. This is possibly why root suppression could not always be reproduced in sand in the present studies.

Daily measurements of elongation of tomato and clover roots in agar showed that there was a greater variability among the unsterile plants than among sterile. The overall effect of micro-organisms was one of stunting but in some instances there appeared to be a temporary stimulation followed by stunting. This could be due to the dynamic and complex balance of micro-organisms in the rhizosphere in which "stimulative" organisms similar to those recorded by Pantos [28] predominated occasionally but more often the stunting organisms were dominant. Stimulation of root growth may be caused by the production of stimulants, removal of inhibitors (shown to be present by Kerr [21] and Eliasson [12]) or partial destruction of these to stimulative levels. Current studies with pure cultures of bacteria and fungi isolated from roots may reveal the mechanisms responsible for stimulation and stunting of roots.

In most experiments the greater numbers of secondary roots on

sterile plants than on non-sterile appears to be due to the greater primary-root length of the former. Non-sterile roots in most experiments had more secondary roots per centimetre of primary root, the differences being significant in some experiments. Inhibition of primary-root growth by the rhizosphere microflora apparently had lessened apical dominance of the primary root allowing more development of secondary root primordia.

Root-hair inhibition with non-sterile clover plants appeared to be caused by specific organisms which occurred at greater concentration than did those causing root stunting. Similarly they occurred in a wide range of soils and the severity of the root hair effect varied with different soil inocula. The inhibition of root hairs by organisms more numerous than those causing root stunting, its strong expression on subterranean clover alone and the marked consistency of the effect in sand culture suggests that root-hair inhibition may be unrelated to root stunting. In no instance was any root-hair stimulation by micro-organisms observed.

Despite significant root-length differences the top weights of the sterile plants were not significantly different from those of non-sterile plants except with radiata pine. The failure of depressed root growth to influence the growth of tops of tomato, clover, and phalaris is probably due to abundant moisture and nutrient supply, but top growth could be affected where these are limiting. In the present studies radiata pine was the only test plant which experienced some moisture stress which may account for the relation between depressed top and root growth in this plant.

Troughton [42] has indicated that availability of water, nutrient supply, acidity, and oxygen levels influence root development. The adequate moisture, nutrients and aeration under the test conditions, together with the observation that the root-stunting organisms were eliminated at dilutions which still permitted the development of an abundant rhizosphere population, indicates that organic substances produced by specific micro-organisms are probably responsible.

Many organic compounds have been recorded to affect root growth. Norman [26] referred to repression of root growth by five antibiotics and of 20 others tested most inhibited barley root elongation. Evidence presented by Brian [6] supporting the occurrence of antibiotics under natural conditions suggests that root stunting

could be caused by antibiotic production in the rhizosphere. Auxins and other growth substances have been shown to be root-growth antagonists or synergists (Åberg [1]). The most prominent of these appear to be indoleacetic acid and 2,4-dichlorophenoxy acetic acid. Studies on root-hair deformation of legumes (Wilson [43], Nutman [27]) indicate the presence of indoleactic acid in the rhizosphere. Anti-auxins such as α-(naphthylmethylsulphide)-propionic acid may counteract auxin depression of root growth and themselves act as stimulants (Åberg [1 2]). Many other organic compounds affect root growth and morphology. Some of these are 2,3,5-tri-iodobenzoic acid (Åberg [2]), amino acids, sulphonamides (Barnes and Naylor [5]) benzene hexachloride and allyl alcohol residues (Persidsky and Wilde [31]), and volatile humus substances (Persidsky and Wilde [30]). Practically nothing is known of the synthesis and utilization of many of these organic substances by micro-organisms in the rhizosphere. Elliasson [12] found that retarded growth of wheat roots could be overcome by application of anti-auxins or of activated charcoal. Since he took no precautions to ensure sterility it is possible that growth-retarding root "exudates" could have originated from a rhizosphere flora. The failure to transmit root stunting to sterile plants cannot be regarded as evidence against the production of root-inhibiting substances by rhizosphere micro-organisms because in these experiments it was unlikely that the concentration of active substances would be similar to that when micro-organisms are actually present on the roots. Further, the substances may be heat labile or of a transient nature, being unstable or readily decomposed by other micro-organisms.

For similar reasons to those for root stunting, root hair suppression appears to be due to specific substances produced by micro-organisms. This is supported by the slight transmission of the root-hair effect by sterilized filtrate after non-sterile growth of clover. Wright [44] recorded a reduction in numbers of root hairs of red clover in agar containing the antibiotic allidin or gladiolic acid while Meesters [25] found root-hair-growth depression by indoleacetic acid at $10^{-5}M$ concentration and Jackson [17] has shown root-hair suppression by 10^{-6} to $10^{-3}M$ concentration and root-elongation suppression at $10^{-7}M$. If root-hair inhibition of subterranean clover occurs at higher concentrations of indoleacetic acid than root-length inhibition, the observation of root-hair inhibition

without root-length suppression would disqualify this as the active root-hair-inhibiting substance. Since even less is known of substances affecting root-hair formation than affecting root growth, further speculation on modes of microbial inhibition of root hairs is unwarranted.

These results have clearly shown that the rhizosphere population can significantly affect the development of roots and root hairs of clover, grass, tomato and pine growing in sand and agar. If experiments in progress indicate that this also occurs in soil then it is obvious that a predominance of micro-organisms causing root and root-hair suppression in the field could materially influence crop yield.

SUMMARY

Subterranean clover, tomato, phalaris, and radiata pine were grown with a complete plant- nutrient solution in sterile sand and agar and inoculated with soil suspensions prepared from unsterilized and from sterilized soil.

The presence of micro-organisms reduced primary-root growth in all plants and total root growth in most plants. The total numbers of secondary roots were lower in non-sterile treatments but there was a tendency for an increase in the concentrations of secondary roots with the non-sterile plants. Under the test conditions only radiata pine grown in sterile sand produced significantly greater top growth than those grown in the presence of micro-organisms. Root-stunting micro-organisms were shown to occur in each of four different soil types used in the studies but the extent of stunting varied with the soil. In agar, root stunting was observed at 5 days and 9 days after planting (and inoculation) with subterranean clover and tomato respectively.

Production and growth of root hairs by subterranean clover was markedly reduced by organisms from all four soils tested, the reduction varying with the soil. In contrast to root-stunting organisms, root-hair suppressing micro-organisms were abundant in soil. Root-hair suppression was apparent in sand after 3 days and is an inhibition of root-hair development rather than microbial digestion of existing root hairs. Only slight root-hair reduction was observed for tomato and phalaris.

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LETTER TO THE EDITORS

Utilization of Soil by the Plant Root System

If the uptake of mineral nutrients by the plant is considered as a proces of extraction of part of the reserve of available substances, attention is directed to some special relationships, which might otherwise be overlooked.

One of the facts demanding closer consideration is the importance of the volume of soil available for rooting. In the field, particularly under many horticultural practices, the plant seldom has enough soil at its disposal to allow optimal development of its root system. Spread of the root system is restricted by competition or by other factors, *viz* shallow soil, pot culture *etc.*

However, often a considerable restriction of soil volume hardly impairs growth of the plant. This suggests that there must be large differences regarding the extent of soil utilization. Some of the investigators first using pot experiments already considered these implications [4]. Kraus [6] concluded that the growth of plants in pots was related to more intensive root functioning.

Attention must also be drawn to the fact that the amount of nutrients absorbed by a crop is in general only a small part of that which is available, even on poor or deficient soils, and especially with the more immobile nutrients.

The conclusion must be that generally the soil is only partly utilized, and that a large proportion of the soil is not being exploited by the roots. Bray [1] has already stressed this fact and related the utilized volumes to differences in mobility of nutrients.

Is it therefore possible to obtain some idea of the net volume of soil utilized in feeding the plant? To be able to do this data are needed on the total root surface and on the distances over which extraction of nutrients can occur.

Since the amount of labour involved in obtaining an estimate of the total root surface is prodigious, information in the literature is scarce. However, some investigators have measured total root length and root diameters and these data can be used for calculations. The objection may be raised that the total root surface is not equivalent to the active absorbing surface. But although at any fixed time this discrepancy exists, in the course of the growth of the root system all parts of it have been young and active at some time. Thus there can be no objection to taking total root surface into account.

A difficulty arises when the distance over which nutrient extraction occurs has to be estimated. Exact measurements have not been made and would be very difficult to obtain. However, a reasonable estimation seems possible. Tepe and Leidenfrost [11] have determined the distances over which several nutrients can be extracted from water-saturated soil by ion-exchange units. Their average values range from 2.5 to 20 mm, according to the mobility. Former experiments [13] suggested that, provided a mantle of soil around the root of about 2 to 3 mm – maximal root-hair length – contained a high amount of nutrient, the root was independent of nutrients supplied by diffusion over greater distances. The conclusion seems justified that at least a mantle of root-hair length thickness around the roots can be fully utilized and is the main source of supply.

In Table 1 the results of the calculations are given.

TABLE 1

Percentage of the total volume of soil occupied by the root system in direct contact with the root and functioning as source of supply of nutrients.

Author	Total soil volume dm^3	Root contact volume dm^3	Supply percentage	Crop
Weaver, Kramer, and Reed [12]	60	0.06	0,1	Winterwheat
Nutman [9]	28.3	1.5	5.3	Coffee
Pavlychenko [10]	150	50	33	Summer rye
Pavlychenko [10]	50	108	216	*Poa pratensis*, sod
Evans [2]	28.3	0.74	2.6	Sugarcane
Kullmann [7]	15	0.09	0.6	Clover
	15	0.58	3.9	*Dactylis glomerata*
	12	0.06	0.5	alfalfa
	12	0.66	5.5	*Festuca ovina*

Although the exact values obtained are debatable and will only relate to the nutrients mainly occurring in the adsorbed state the results are certainly of value. With one exception, all percentage values lie far below 100 per cent. Most values do not even attain 5 per cent. This certainly implies that the interpretation of restricted soil utilization under normal circumstances is valid. Also that restriction of the volume of soil for the growth of the plant often does not impair growth. It will be clear that here a favorable structure is of utmost importance to allow the more intensive permeation of soil by the roots.

The very high value (216 per cent) of a 5-cm layer of grass sod, suggests that here a complete utilization of this shallow layer can occur.

In the literature on uptake a few data can be found which are in accordance with the interpretation given above. Mitscherlich [8] noticed that in Neubauer tests the phosphate uptake by the seedlings was not proportional to the amount of soil used. Using 25 g of soil the phosphate uptake was 1.3 mg. When using 100 g of soil the uptake only increased to 2.5 mg, instead

of rising to 4 × 1.3 mg. Phosphate extraction is therefore better from the smaller amount of soil.

In the book Physiology of Trees, edited by Kramer and Kozlowski [5], the following reference is found. "Büsgen and Münch (1931) cite Nobbe as stating that the ability of pine to succeed where silver fir and spruce failed is the result of the pines having 24 times as many root branches and tips and 8 times the absorbing surface of the other species".

From recent research on uptake of phosphate by Fried *et al.*[3] we can quote: "According to the present experiments the rate of phosphorus release by the soil is at least 250 times as great as the rate of plant uptake. This effective soil volume would, therefore, have to be less than 0.4 per cent of the total soil mass". If the potential release rate is faster than uptake then we may conclude that the rate of diffusion between the desorption surface and the absorbing root surface is limiting. This will be the case if the soil is not in the immediate vicinity of the root. The "effective soil volume" can be interpreted as the thin mantle of soil covering the root system.

So the conclusion may be drawn that the volume of soil mainly contributing to the nutrition of the plant in the field is much smaller than the total volume occupied by the roots. An exception must be made for the highly mobile nutrients such as nitrate, which will be drawn towards the root surface along with the extracted water.

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LETTER TO THE EDITORS

Effective Nodulation of *Lupinus digitatus* by Native Rhizobia in South-Western Australia

Introduction

Several species of *Lupinus* are cultivated in South-Western Australia, where the genus is exotic. Gladstones [3 4] has discussed them in detail, noting that *L. digitatus* Forsk. has naturalized in Western Australia along a 400-mile coastal belt of sandy soils. This species, known there as the "West Australian blue lupin", has naturalized to a much greater extent than have any of the other introduced lupin species.

In 1930 Adams and Riches [1] found that *L. digitatus*, as opposed to other lupins they investigated, was susceptible to nodulation by the local soil rhizobia. Lange and Parker [5] recently confirmed that *L. digitatus* is very susceptible to nodulation in the field when compared to *L. angustifolius* L. and *L. luteus* L. Lange [6], with pure cultures of bacteria, has demonstrated that many indigenous rhizobia can produce root nodules on *L. digitatus*, as well as on a number of other introduced legumes. In the latter experiments nodules formed on *L. digitatus* frequently showed the presence of leghaemoglobin, which suggested that effective symbiosis between this lupin and native rhizobia might be common. The present study was therefore undertaken to assess the *effectiveness* on *L. digitatus* of some selected strains of rhizobia from a range of legumes indigenous in South-Western Australia.

Methods

Thirty-nine strains of "native" rhizobia from 39 host species in 22 genera of the legumes were tested for their symbiotic performance on *Lupinus digitatus*. The serial numbers of the bacterial strains used, with their hosts of isolation are as follows: 14, *Acacia diptera* Lindl.; 54, *A. ericifolia* Benth.; 121, *A. extensa* Lindl.; 126, *Aotus Preissii* Meissn.; 134, *A. villosa* Sm.; 88, *Bossiaea eriocarpa* (R. Br.) Benth.; 92, *B. Preissii* Meissn.; 110, *B. Webbii* F. v. M.; 91, *Brachysema lanceolatum* Meissn.; 120, *Chorizema aciculare* (D.C.) C.A. Gardn.; 119, *C. cytisoides* Turcz.; 90, *C. Dicksonii* Grah.; 101, *Daviesia brevifolia* Lindl.; 57, *D. hakeoides* Meissn.; 98, *Dillwynia uncinata* (Turcz.) C.A. Gardn.; 62, *Euchilopsis linearis* (Benth.) F.v.M.; 87, *Eutaxia epacridioides* Meissn.; 105, *E. virgata* Benth.; 100, *Gastrolobium trilobum*

Benth.; 106, *G. villosum* Benth.; 60, *Gompholobium marginatum* R. Br.; 111, *G. polymorphum* R. Br.; 19, *Hardenbergia Comptoniana* (Andr.) Benth.; 26, *Hovea chorizemifolia* D.C.; 118, *H. pungens* Benth.; 16, *Isotropis cuneifolia* (Sm.) Domin.; 125, *Jacksonia furcellata* (Bonpl.) D.C.; 102, *J. spinosa* (Labill.) R. Br.; 77, *Kennedya coccinea* Vent.; 73, *K. eximia* Lindl.; 2, *K. prostrata* R. Br.; 133, *Latrobea hirtella* Turcz.; 89, *Mirbelia floribunda* Benth.; 127, *M. spinosa* Benth.; 17, *Oxylobium capitatum* Benth.; 27, *O. parviflorum* Benth.; 84, *Pultenaea reticulata* (Sm.) Benth.; 59, *Sphaerolobium alatum* Benth.; 93, *Viminaria denudata* Sm.

The experiment was conducted in a closed glasshouse room with adequate sunlight and pressurised by evaporator coolers delivering washed air. The floors, walls, ceilings, and benches of the room were sprayed with insecticidal, antifungal, and antibacterial sprays, and with an emulsion of 25 per cent paraffin oil in water at the rate of 15 litres per 1,000 square feet of surface. Dust immobilisation was maintained by further paraffin oil sprayings as required.

Plants were grown in open, porous draining pots, each containing 2 kg dry, yellow subsoil sand. These were dressed with a nutrient mixture providing major and minor elements, brought to field capacity, wrapped, and sterilised.

Seeds of *L. digitatus* were surface disinfected, germinated under sterile conditions, planted aseptically, and inoculated by sterile pasteur pipette with a water suspension of the rhizobial strain under test. Four germinated seeds were planted per pot. Two lupin isolates, D25 and D27, of known effective performance, were included in the experiment for comparison. No-nitrogen (–N) and plus-nitrogen (+N) treatments were used, both being uninoculated. The +N treatment received 150 ppm of NH_4NO_3 at seeding and this dressing was repeated at 14 and 28 days. There were three replicate pots for each treatment, except in the —N treatment where 24 pots were used. All pots were randomized within a single block and were watered with sterile distilled water each day.

The experiment was terminated at 50 days. Plants were first ranked visually on a 3-point scale on the basis of colour and size. The tops were then harvested, and the tops of the four plants from each pot were bulked and dried. Root systems were washed and inspected for incidence and type of nodulation. Nodules were sectioned fresh and ranked visually on a 6-point scale on the basis of leghaemoglobin colour. The bulked tops from each pot were weighed from a dessicator, and ground. Total nitrogen determinations were made on the material from each pot, using the method of Willits, Coe, and Ogg [8].

Results

Nodules were present on all inoculated plants, and on 14 of the 96 —N uninoculated plants. The latter were few, small, and with a leghaemoglobin rating of only 1 or 2. They represented some uncontrolled nodulation, but this did not detract from the value of the experiment as a measure of the

TABLE 1

Comparison of inoculant treatments on the basis of total nitrogen in the tops. Any two means *not* bracketed by the same line are significantly different at the level indicated

Strain No.	Mean total nitrogen (mg per pot)	Significant differences	
		$P = 5\%$	$P = 1\%$
D. 25	148.7		
*+N	94.5		
D. 27	87.4		
125	83.8		
16	79.4		
126	71.4		
111	58.5		
134	50.5		
133	46.1		
127	45.8		
73	44.8		
100	42.4		
62	41.8		
91	41.1		
102	40.1		
77	37.0		
118	36.1		
17	35.5		
84	35.3		
26	35.3		
59	34.9		
120	34.5		
93	34.3		
119	33.8		
27	33.3		
121	33.1		
92	33.1		
105	32.7		
2	32.2		
*–N	31.4		
89	31.2		
101	31.0		
14	30.0		
87	29.9		
106	29.0		
110	28.9		
54	28.9		
57	28.4		
90	27.8		
60	27.6		
19	27.0		
88	26.6		
98	25.3		

*: uninoculated controls

relative *effectiveness* of the bacterial strains. The —N uninoculated control plants were used as the baseline for comparisons of effectiveness.

Mean values for the total nitrogen in the plant tops from each treatment were calculated. A statistical analysis performed to determine significant differences between treatment means, on the basis of the total nitrogen present in the tops from each pot, is summarized in the range-test diagram of Table 1.

The regressions of dry weight of tops, vigour ranking, and visual haemoglobin ranking upon the total nitrogen of the tops were determined. The correlations are given in Table 2.

TABLE 2

Correlation coefficients (r) of dry weight, leghaemoglobin ranking and vigour ranking on total nitrogen in the tops of *Lupinus digitatus*.

Total nitrogen in the tops per pot	Dry weight of tops per pot	r	d.f.
		0.90	42
,,	Leghaemoglobin ranking	0.76	40
,,	Vigour ranking	0.89	39

Discussion

Symbiosis between introduced legumes and nodule bacteria from indigenous legumes has not often been reported. McKnight and Bowen [2], in Queensland, also found examples of such symbiosis, and regarded the "native" rhizobia as playing a role in the establishment of introduced tropical legumes.

In the present study it has been established that some of the rhizobia associated with indigenous legumes in South-Western Australia are capable of effective symbiosis with *L, digitatus,* and that these bacteria range from fully effective through partially effective to ineffective on that lupin.

Isolates from *Jacksonia furcellata* (125), *Isotropis cuneifolia* (16) and *Aotus Preissii* (126) were the most effective of the native strains when inoculated onto *L. digitatus*. The lupin strain D25 fixed more nitrogen than was present in the tops of the +N controls, but the nitrogen in the tops of the plants nodulated by D27 was not significantly less than that in the +N control plants (see Table 1). Three of the 39 "native" strains of bacteria were not significantly different from the lupin strain D27; 5 strains were better than —N control at the 5 per cent level of probability and 4 strains at the 1 per cent level.

Several points of interest arise from this work and from a related study already reported [5]:

(i) *Effective* symbiosis occurs between *L. digitatus*, of Mediterranean origin, and nodule-bacteria from legumes indigenous to a geographically isolated and remote continent. Moreover ,the genus Lupinus is placed in the Tribe Genisteae whereas Aotus, Jacksonia and Isotropis are all placed in the Tribe Podalyrieae. There is thus no obvious relationship between the two groups except their common membership of the sub-family Papilionatae.

(ii) Two other mediterranean lupins, *L. luteus* and *L. angustifolius*, nodulate infrequently in the same environment where *L. digitatus* nodulates freely.[5] In pot experiments these two lupins did not nodulate with *any* of the pure culture isolates of "native" nodule bacteria, most of which nodulated *L. digitatus* [6].

(iii) Lupin nodule bacteria from each of the three lupin species showed no host specificity when inoculated on to the same three species [5]. It appears, then, that *L. digitatus* possesses some special quality making for susceptibility to nodulation by nodule bacteria.

The evidence indicates that effective nodulation of *L. digitatus* by native rhizobia could be an important factor contributing to its naturalisation in South-Western Australia. It also helps to account for the relative ease with which this lupin may be established as a pasture plant on the virgin sandy soils of the coastal region.

The regressions of dry weight, vigour ranking, and leghaemoglobin ranking on total nitrogen in the tops show that any one of these quick measures is useful in estimating symbiotic effectiveness in the lupin.

Summary

Thirty-nine nodule bacteria isolated from indigenous legumes in South-Western Australia were found to range in effectiveness in symbiosis with *Lupinus digitatus*. Effective nodulation by bacteria from some of the native legumes is, therefore, probably involved in the successful naturalization of this introduced lupin.

It was found that dry weights, vigour ranking and ranking visually for leghaemoglobin concentration, are all useful in the rapid assessment of bacterial strain performance in nitrogen fixation in the lupin.

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